| (51) International Patent Classification 6 :

 C12N 5/06, A61K 35/28, C12Q 1/68, G01N 33/577 // A61K 48/00 | A1 | (11) International Publication Number: **WO 98/12304**

 (43) International Publication Date: 26 March 1998 (26.03.98) |

(21) International Application Number: PCT/GB97/02549

(22) International Filing Date: 19 September 1997 (19.09.97)

(30) Priority Data:

9619597.9	19 September 1996 (19.09.96)	GB
60/025,747	19 September 1996 (19.09.96)	US
60/027,735	3 October 1996 (03.10.96)	US
08/933,600	18 September 1997 (18.09.97)	US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CULTURE SYSTEM FOR HEMATOPOIETIC STEM CELLS

(57) Abstract

The invention relates to an *in vitro* culture system for the expansion of mammalian hematopoietic stem cells (HSCs).

CULTURE SYSTEM FOR HEMATOPOIETIC STEM CELLS

FIELD OF THE INVENTION

The invention relates in general to providing a culture of hematopoietic stem cells (HSCs).

BACKGROUND

Hematopoiesis is the process of generating mature blood cells. The adult hematopoietic system of mammals is a dynamic hierarchy of cells with the HSC at its foundation, in that the HSC gives rise to all other cell types of the hematopoietic system. HSCs are rare and generally comprise less than approximately 0.01% of any hematopoietic cell culture.

The source of hematopoietic cells, namely HSCs, and the processes involved in the differentiation of the various hematopoietic cell types from the HSCs, are of considerable interest as they may provide useful tools for therapy, such as bone marrow transplants, gene therapy and for the production of blood related proteins.

Dexter and LTC-IC culture systems are able to maintain HSCs; however, cultures of bone marrow or fetal liver cells do not increase HSC numbers *in vitro*. Hematopoietic cell cultures are difficult to maintain and require complicated media. A review of the problems and difficulties of hematopoietic cell cultures is provided by McAdams *et al.* (1996, Tibtech, 341-349).

Due to the heterogeneity of the prior art culture systems, such as the Dexter and LTC-IC culture systems, it is difficult to manipulate the rare HSCs. There is a need in the art for a source of inducible and/or expandable, homogeneous hematopoietic stem cells. In order to improve clinical applications in hematopoietic transplantation related to the treatment of genetic and infectious disease of the blood, and the elimination and replacement of leukemic cells.

The development of hematopoietic precursors has been studied using the mouse as a model, where it has been found that the number of definitive HSCs is substantially constant

from day 14 in gestation throughout the entire life span of the adult.

Hematopoietic events have been determined to begin in the yolk sac (YS) at day 7.5 in gestation (Russell and Bernstein, 1966, "Blood and blood formation" in Biology of the Laboratory Mouse, Second Edition, E.L. Green, ed., McGraw-Hill, New York, pp. 351-372) and then shift to the fetal liver and later to the spleen and bone marrow (Moore and Metcalf, 1970, Br. J. Haematol., 18: 279-296; Johnson and Moore, 1975, Nature, 258: 726-728). It was generally accepted that this picture reflected the consecutive migration of HSCs from the YS to the definitive hematopoietic territories. Although primitive hematopoiesis and committed hematopoietic progenitors can be detected in the YS as early at days 7-8.5 post coitum (p.c.) (Moore and Metcalf, 1970, supra; Johnson and Barker, 1985, Exp. Hematol., 13: 200-208; Wong et al., 1986, Proc. Natl. Acad. Sci. USA, 83: 3851-3854; Liu and Auerbach, 1991, Development, 113: 1315-1323; Cumano et al., 1993, Proc. Natl. Acad. Sci. USA, 90: 6429-6433; Palacios and Imhof, 1993, Proc. Natl. Acad. Sci. USA, 90: 6581-6585; Huang and Auerbach, 1993, Proc. Natl. Acad. Sci. USA , 90: 10110-10114), the lack of definitive colony-forming-unit spleen (CFU-S) progenitors and HSCs in the YS until late day 9 post-coitum (p.c.) (Sonoda et al., 1983, Dev. Biol., 97: 89-94; Perah and Feldman, 1977, J. Cell Physiol., 91: 193-200; Symann et al., 1978, Expl. Haematol., 6: 749-759; Samoylina et al., 1990, Sov. J. Dev. Biol., 21: 127-133; Medvinsky et al., 1993, Nature , 364: 64-67) and day 11 p.c. (Moore and Metcalf, 1970, supra; Müller et al., 1994, Immunity, 1: 291-301) respectively brought this widely held dogma into controversy (reviewed in Medvinsky, 1993, Sem. Dev. Biol., 4: 333-340; Dzierzak and Medvinsky, 1995, Trends in Genetics., 11: 359-365).

Recently, in the mouse embryo a pre-liver intraembryonic site of potent definitive hematopoietic activity has been identified. This axial, mesodermally derived region of the mouse embryo, containing the dorsal aorta, genital

ridge/gonads and pro/mesonephros (AGM), has been shown to
harbor adult type multipotent hematopoietic progenitors
(CFU-S) and totipotent long term repopulating hematopoietic
stem cells (HSCs).

Comparisons of activity harbored in this intraembryonic
hematopoietic site with that in the YS has revealed several
differences in dynamics as well as potency. While CFU-S
progenitors appear simultaneously in the YS and the AGM
region at late day 9 p.c. (Medvinsky et al., 1993, supra),
temporal quantitation studies comparing the number of CFU-S
progenitors in the YS, AGM region and fetal liver have
revealed that 1) CFU-S progenitor number and frequency in
the AGM region greatly exceeds that in the YS from day 9
p.c. to late day 10 p.c. and 2) CFU-S progenitor numbers and
frequencies decrease dramatically in the AGM at day 11 p.c.
while there is a concomitant increase in liver CFU-S
(Medvinsky et al., 1993, supra). Furthermore, definitive
HSC activity has been found in the AGM region at late day 10
p.c., at a time slightly earlier than in the YS and fetal
liver (Müller et al., 1994, supra). While these results
indicate that the intraembryonic AGM region is the most
potent pre-liver site of definitive hematopoietic activity,
the direct measurement of CFU-S and HSCs within different
parts of the embryo as a means of identifying the primary
source of definitive hematopoietic activity is not adequate
due to the early establishment of the vasculature between
the yolk sac and the embryo body, and the active interchange
of cells via the circulation and possible interstitial
migration as was suggested in amphibian embryos (Turpen and
Knudsen, 1982, Dev. Biol., 89: 138-151).

Previously, the ontogenic source of the definitive adult
hematopoietic system has been determined in non-mammalian
vertebrates by orthotopic embryo grafting experiments
(reviewed in Dieterlen-Lievre and Le Douarin, 1993, Sem. in
Devel. Biology, 4: 325-332). Two sites of hematopoiesis
have been found to exist early in the embryonic development
of birds and amphibians. In the avian species, it was found
in chick-quail (Dieterlen-Lievre, 1975, J. Embryol. Exp.

Morphol., 33:   607-619) and chick-chick embryo grafts
(Lassila et al., 1978, Nature, 272:   353-354) that the
intra mbryonic region containing the dorsal aorta is
responsible for definitive hematopoiesis while the YS
produces only transient embryonic hematopoiesis. Similarly,
in amphibians in grafts between the intraembryonic dorsal
lateral plate and the ventral blood islands (YS analogue),
it was found that the intraembryonic region containing the
pronephros is the major source of definitive hematopoiesis
(Turpen et al., 1981, Dev. Biol., 85:   99; Turpen and
Knudsen, 1982, supra; Kau et al., 1983, J. Immunol., 131:
2262-2266; Maeno, et al., 1985, Dev. Biol., 110:   503-508).
While the orthotopic embryo grafting method has yielded
conclusive results in these non-mammalian vertebrates, the
in utero development of the mouse embryo prohibits such
experiments at the present time and requires alternative
methods of investigation.

There is a need in the art to provide a renewable cell
population of hematopoietic stem cells (HSCs) and methods of
generating and expanding HSC progenitor cells.

SUMMARY OF THE INVENTION

The invention is based on the development of an in vitro
culture system, and on the discovery that HSC activity is
initiated autonomously in the mammalian AGM prior to the
appearance of stem cells in the yolk sac and prior to their
appearance in the liver.   The invention thus provides
hematopoietic stem cells and their progenitor cells (i.e.,
generated in the AGM region) which are less mature than
previously-identified stem cells, and which appear to be the
founder cells for the differentiated adult blood system and
which colonize the other hematopoietic tissues.

An "HSC" is herein defined as a pluripotent precursor cell
which gives rise to substantially all hematopoietic cell
types and has the ability to repopulate the hematopoietic
system of a lethally-irradiated mammalian recipient in the
long term (that is, longer than 4 months, preferably longer
than 8 months and most preferably during the lifetime of the

recipient); such a cell recipient may be an adult mammal or, alternatively, may be a neonatal mammal. Cell types to which HSCs give rise include, but ar not limited to, erythrocytes, granulocytes, B- and T-cells, macrophages, megakaryocytes, eosinophils, mast cells, dendritic cells, neutrophils, basophils and N-K cells. Totipotent precursor cells give rise to all such cell types in the sense that they reconstitute the hematopoietic system.

As used herein, the term "cells" is defined as including dissociated cells, intact tissue or tissue fragments.

As used herein, the term "tissue" refers to intact tissue or tissue fragments, such that the cells are sufficiently aggregated (associated) so as to form a cohesive mass.

As used herein, the term "mammal" is defined as any member of the Class Mammalia.

The invention thus recognizes the importance of the mammalian AGM region for generation of- and increase in HSCs. The invention therefore provides a microenvironment for the generation, induction and/or expansion of clinically-useful HSCs.

As used herein, "generation" is defined to mean the conversion of pre-HSCs to HSCs.

As used herein, the term "pre-HSC" is defined to mean a cell that can be induced to give rise to HSCs.

As used herein, "induction" is defined to mean initiation of division of a quiescent, undifferentiated cell and/or of its development to a more differentiated state.

As used herein, the term "AGM" is defined as a pre-liver intraembryonic site of potent definitive hematopoietic activity which is an axial, mesodermally derived region of the mammalian embryo, containing the dorsal aorta, genital ridge/gonads and pro/mesonephros (AGM) and surrounding mesenchymal tissue.

Furthermore, the invention also is based on the discovery that the HSC activity generated in the AGM region on culture is at least 15-fold more abundant than in uncultured tissue. HSC activity generated in the AGM region on culture may be 50-fold, 100-fold and 200 fold more abundant than in

uncultured tissue. This increase may be the result of 1) the proliferation/expansion of one or a few (1-10) starting HSCs and/or 2) the developmental induction of a pool (2-50) f pre-HSC cells.

The HSCs also enable HSC-expansion/induction factors to be identified and cloned using standard techniques such as the phage antibody display system, the RT-PCR differential display method and representational display analysis (RDA) and also enables the identification of cellular components of the AGM to provide a microenvironment which supports HSC generation, expansion and/or induction.

The present invention provides an *in vitro* culture system for the generation or expansion of inducible and/or expandable mammalian hematopoietic stem cells (HSCs), comprising cells of the AGM of an embryonic mammal and nutrients sufficient to support the generation and/or expansion of mammalian HSCs, and containment therefor. Particularly preferred mammals include mice and humans. It is generally recognized in the art that the human hematopoietic system is substantially analogous with the mouse hematopoietic system, and therefore the present invention is directly applicable to human HSCs.

It is preferred that the cells of the culture system comprise tissue.

It is additionally preferred that the cells reside on a solid support.

As defined herein, a "solid support" refers to any support used for culturing cells such as filters and beads, provid d the support is permeable.

The culture may contain a variety of hematopoietic cell types including colony-forming-unit spleen cells (CFU-S), which also may be expanded in the culture. A "CFU-S" is herein defined as a multipotent progenitor cell which gives rise to colonies containing erythroid and myeloid cells.

It is preferred that the *in vitro* culture system comprises CFU-S cells.

The term "expandable" is used to indicate that the number of HSCs introduced into or originating in the culture can be

increased in the culture by at least 10-fold and preferably, at least 15-fold. An increase in the number of HSCs is determined by measuring an increase in HSC activity by testing for *in vivo* long-term reconstitution of the hematopoietic lineage of a lethally irradiated mammalian recipient.

As used herein, the term "inducible" is used to indicate that HSCs, pre-HSCs or stromal cells can be stimulated t produce particular proteins (e.g. growth factors or receptors) and/or form particular hematopoietic cell types. Inducible also may refer to stimulation of cells of the AGM to produce particular proteins.

As used herein, the term "cells of the AGM" is defined to refer to any sub-population of AGM cells which can provide a supportive microenvironment for HSC generation, expansion or induction.

As used herein, the term "exogenous" cells refers to cells taken from a source that is outside of the place to which they are returned; for example, exogenous may refer to cells taken from a first mammal or tissue or culture thereof and placed into a second mammal or tissue or culture thereof, or may refer to cross-species cell transplants. Similarly, "endogenous" refers to cells that have arisen in- and continuously occupied the tissue in which they are found without ever having undergone removal from- and replacement to that tissue.

As used herein, the phrase "enriched culture" refers to an increased number of HSCs relative to the other cells present in the culture when compared to a corresponding non-cultured AGM from which the HSCs and/or pre-HSCs were derived. Preferably, the enriched culture has an increased number of HSCs (e.g. at least 15-fold) relative to other cells present in the culture when compared to the *in vitro* culture of inducible and/or expandable HSCs of the present invention.

Preferably, the AGM is from a first mammal and the HSCs and/or pre-HSCs are from a second mammal.

It is contemplated that the first and second mammals are a single individual of a single species; it is further

contemplated that the first and second mammals are different individuals of a single species and the AGM of the first mammal substantially lacks endogenous HSCs and pre-HSCs prior to the introduction of HSCs and/or pre-HSCs from the second mammal in culture; it is also contemplated that the first and second mammals are different individuals of two different species and the AGM of the first mammal substantially lacks endogenous HSCs and/or pre-HSCs prior to the introduction of HSCs and/or pre-HSCs from the second mammal in culture.

In a preferred embodiment, the first mammal is a non-human mammal, the second mammal is a human, and the AGM of the first mammal substantially lacks endogenous HSCs and/or pre-HSCs prior to the introduction of human HSCs and/or pre-HSCs in culture.

Preferably, the first mammal is immune-deficient.

It is also preferred that HSCs and/or pre-HSCs of the second mammal are transfected with a gene of interest. According to the invention, the HSC will give rise to all the lineages of hematopoietic cells each containing a copy of the introduced gene. Particularly useful genes include human β-globin for thalassemias, adenosine deaminase (ADA), glucocerebrosidase for Gaucher's disease, Bruton's trypsin kinase for Crigler najjar syndrome, antiviral constructs, ribozymes and gene repair enzymes.

The invention also encompasses an *in vitro* culture method, comprising culturing cells from the AGM of a mammalian embryo *in vitro* under conditions which permit generation and/or expansion of HSCs.

Preferably, the cells are cultured on a solid support.

It is also preferred that the cells comprise tissue.

Preferably, the method further comprises before the culturing step, the step of substantially purging the cells of the AGM of endogenous HSCs and pre-HSCs, contacting the purged cells with exogenous HSCs so as to form a mixture of cells, and culturing the mixture of cells.

It is preferred that the culturing step permits aggregation of the cells to form tissue.

It is additionally preferred that the method further comprises the step after culturing of dissociating the cells of th tissue.

It also is preferred that after the dissociation step, the method further comprises the step of sorting HSCs from the dissociated cells, although it is possible to inject the entire dissociated cell population into a recipient mammal without such sorting.

It is preferred that the tissue is cultured at the air/medium interface.

As used herein, the term "air/medium interface" is defined as referring to the boundary between the cell culture growth medium and an ambient gas which contacts that medium one or more of its boundaries. This term is used herein interchangeably with "air/liquid interface". Such an interface may be found on or within 5-10 mm of the upper surface of a pool of culture medium in a container, such as a petri dish or tissue-culture flask. In particular, the air/medium interface may occur at a boundary of a solid support on which cells of the present invention are grown, wherein such a support is saturated or perfused with culture medium and one or more surfaces of the support are in contact with an ambient gas. An ambient gas may be atomospheric air. Alternatively, it may be either a homogeneous- or a heterogeneous population of atoms or molecules which are found in the gaseous phase and which are represented in a concentration and proportion relative to one another that will support the survival and proliferation of mammalian cells.

It is also preferred that the tissue is cultured at 37°C, under 5% CO_2, in myeloid long-term culture medium supplemented with hydrocortisone succinate to a concentration of approximately 10^{-5} to 10^{-6} M. A highly preferred concentration of hydrocortisone succinate is 10^{-5} M.

In a particularly preferred embodiment, the AGM is taken from a mammal during the stage at which hematopoietic stem cells are maximally proliferative in that tissue. In the

mouse embryo, this stage is between day 10 and 13 p.c., and more preferably 10 days p.c. or at a comparable stage in another mammal. In the human embryo, a pr ferred equivalent stage is the fifth week of gestation. Appropriate stages of AGM development in other mammalian species may be determined by the methods described herein below.

It is preferred that the AGM comprises the anterior section of the AGM. The "anterior section" of the AGM is defined as containing the pronephros, part of the mesonephros, the dorsal aorta, genital ridge/gonads and surrounding mesenchymal tissue. The "posterior section" of the AGM is defined as containing part of the mesonephros, the dorsal aorta, the genital ridge/gonads and surrounding mesenchymal tissue.

Another aspect of the present invention is a method of treating a mammal having a deficiency of HSCs, comprising the steps of culturing cells of the AGM of an embryonic mammal in vitro under conditions which permit generation and/or expansion of HSCs to produce HSCs, and administering the HSCs so generated or expanded to a mammal in need thereof. Causes of HSC deficiency suitable for treatment include, but are not limited to, leukaemia, sickle cell anaemia, thalassaemias, immune deficiencies, depletion of blood cells through accidental radiation poisoning or as a result of radical medical intervention (such as radiation therapy or chemotherapy for leukemia or other cancers), lysosomal storage diseases and viral diseases and infections.

Preferably, the cells of the AGM which are cultured comprise tissue.

The present invention additionally encompasses a method for identifying receptors on the surface of HSCs, comprising generating and/or expanding HSCs using either the culture system or the culture method described above, and making a comparison of the receptors present thereon with those on cells of the hematopoietic system other than HSCs.

Preferably, the comparison is made using the phage antibody display assay.

Another aspect of the present invention is a method for identifying factors produced by HSCs, comprising generating and/or expanding HSCs using either the culture system or the culture method described above, and making a comparison of the factors produced thereby with those produced by cells of the hematopoietic system other than HSCs.

It is preferred that the comparison is performed using RT-PCR differential display assay of representational display analysis (RDA).

Further features and advantages of the invention will become more fully apparent in the following description of the embodiments and drawings thereof, and from the claims.

DRAWINGS

The present invention is now described by way of example only with reference to the accompanying drawings in which:

Figure 1 is a schematic representation of the culture system and the assay of cells generated by this system for HSC and CFU-S activities in recipient mice.

Figure 2 shows the results of an analysis of CFU-S activity in cultured embryonic tissues.

Figure 3 shows a histogram analysis of CFU-S generation potential in cultures and directly transplanted uncultured day 10 p.c. and day 11 p.c. embryonic tissues (for design of the experiment see Figure 1).

Figure 4 shows peripheral blood and multilineage PCR analysis of transplant recipients receiving day 10 p.c. cultured AGM region.

Figure 5 shows PCR analysis of recipient peripheral blood for donor cell engraftment by cultured day 11 p.c. embryonic tissues.

Figure 6 shows PCR analysis of peripheral blood DNA from transplant recipients receiving cultured day 10 p.c. anterior or posterior AGM region.

Figures 7A to 7D show the results of a cell sorting experiment.

Figure 8 shows c-kit expression analysis in AGM and liver

cell suspensions from day 10, 11 and 12 p.c. embryos.

Figure 9 shows cell sorting analysis of cells from the AGM and from the liv r

Figure 10 shows cytospin preparations of AGM c-kit$^+$ sorted cells (top panel) and preparations of liver c-kit$^+$ sorted cells (lower panel).

Figure 11 shows the results of a transplantation experiment performed with Mac-1$^+$ and Mac-1$^-$ sorting criteria.

Figure 12 shows a Southern blot derived from genomic DNA of mouse peripheral blood prepared from recipients of short-term chimeric AGM/HSC cultures one month after transplant.

Figure 13 shows a Southern blot derived from genomic DNA of mouse peripheral blood prepared from recipients of long-term AGM/HSC cultures one month or three months after transplant.

DESCRIPTION

The invention is based on the discovery that hematopoietic stem cell activity is initiated autonomously in the AGM at day 10 of gestation in the mouse embryo, and thus recognizes the importance of the AGM region at day 10 for generation of HSCs. An culture system has been developed *in vitro* which provides a microenvironment for induction and expansion of HSCs. The invention provides a culture of HSCs which are at least 15-fold more abundant than in uncultured tissue, and even at least 100- to 200-fold more abundant.

Using the *in vitro* culture system described herein, the yolk sac, AGM region and the liver were individually cultured in isolation of the other tissues to determine which tissue is the generator of HSCs. The AGM was found to initiate autonomously HSC activity at day 10 in gestation. This is one day prior to the appearance of stem cells in the yolk sac and two days prior to the liver, suggesting that the hematopoietic stem cells generated in the AGM region are indeed the founder cells for the definitive adult blood system and that these cells colonize the other hematopoietic tissues. By providing an expanded culture of HSCs, the invention also provides for identification and cloning of HSC expansion/induction factors, using standard techniques

such as the phage antibody display system, the RT-PCR differential display method and representational display analysis (RDA).

The invention is illustrated by the following nonlimiting examples wherein the following materials and methods are employed to carry out the invention. Example 1 presents data that establish that tissue of the AGM has the capacity to induce and promote the expansion of HCSs in culture. Example 2 describes the characterization of HSC cell surface markers which can be used to identify and enrich in a population cells that are of use according to the methods of the invention. Example 3 presents a method by which growth factors and signalling molecules that mediate hematopoietic cell proliferation and differentiation can be identified. Example 4 describes a chimeric culture in which donor cells comprising HSCs are co-cultured with recipient AGM tissue that has been purged of hematopoietic precursors. Example 5 describes the transfection of HSCs obtained according to the methods of the invention with a gene of interest prior to transplantation of the cells into a recipient mammal. Example 6 presents methods by which a human hematopoietic stem cell population may be generated and/or expanded in AGM tissue or cells. Example 7 presents long-term culture of HSCs in AGM tissue. Example 8 describes methods by which dissociated or cloned cells of the AGM may be prepared for HSC culture.

<u>Preparation of a mammalian culture according to the invention.</u>

As used herein, the term "AGM" is defined as a pre-liver intraembryonic site of potent definitive hematopoi tic activity which is an axial, mesodermally derived region of the mammalian embryo, containing the dorsal aorta, genital ridge/gonads and pro/mesonephros (AGM). An culture derived from mouse AGM is described below. In order to identify th AGM of a mammal other than the mouse, it is necessary to perform temporal staging experiments, e.g. by staining variously aged embryos with an antibody directed against an HSC-specific marker, such as CD34. Morphologically, AGM

tissue is very similar among mammals, facilitating such an analysis.

Preparation of AGM to support expansion of mouse and human HSCs

Early hematopoietic and non-hematopoietic tissues from embryos are used for cultures (see below and Figure 1). The AGM region, YS, liver, head/heart, as well as other pooled tissues (body remnants) are dissected from day 9, 10 and 11 p.c. mouse embryos and cultured in L-15 medium supplemented with 5% fetal calf serum. Embryo age is determined starting with day 0 p.c. on the morning of vaginal plug discovery. When necessary, somite pairs are counted to determine more accurately embryo age. Following culture, the embryonic tissues are treated with 0.04% collagenase, pipetted into a single cell suspension, counted and transplanted intravenously into irradiated (1000 rads of a ^{60}Co source) adult female mice. This procedure is as described earlier (Müller et al., 1994, supra) except that no supporting cells are injected. Mice are housed in positive pressure cabinets and receive neomycin (0.16 g/100 ml) with drinking water for the first month following irradiation and transplantation. Animals are obtained from the specific pathogen-free breeding facilities.

Day 9, 10 and 11 p.c. tissues (AGM, YS, liver, head/heart, body remnants) from mouse embryos are explanted separately onto Durapore 0.65 micron filters (Millipore) supported by stainless steel mesh stands at the air-liquid (or "air-medium") interface (see Figure 1). The filters are washed and sterilized in several changes of boiling water before use in culture experiments. Tissues are cultured at 37°C, 5% CO_2 in myeloid long-term culture media (Alpha MEM, horse serum 12.5%, fetal bovine serum 12.5%, 2-mercaptoethanol 10^{-4}M, i-inositol 0.2mM (optional), folic acid 20μM (optional) (Stem Cell Technologies Inc., Toronto) supplemented with hydrocortisone succinate (Sigma) to yield a final concentration of 10^{-6} M. Following 2-3 days in culture, the tissues are dissociated with 0.04% collagenase, cells counted and transplanted intravenously into irradiated adult

recipients as previously described (Medvinsky et al., 1993, supra; Müller et al., 1994, surpa) for CFU-S and long-term repopulation (HSC) assays. For HSC activity assay, the sex of the embryos is determined during the culture period by PCR analysis using oligos which amplify a 342 base pair fragment of the YMT 2/B gene on the Y chromosome, and only male embryonic tissues are transplanted. The oligonucleotide primer sequences used are:

YMT2-1   CTG GAG CTC TAC AGT GAT GA   [SEQ ID NO: 1]
YMT2-2   CAG TTA CCA ATC AAC ACA TCA C [SEQ ID NO: 2]

Staging to determine human AGM

In humans, yolk sac hematopoiesis begins in the middle of the third week of gestation and decreases from week 5 until week 7 when it disapears. It is thought that yolk sac hematopoietic cells colonize the fetal liver. At week 5, yolk sac BFU-Es (burst forming units-erythroid) decrease in frequency while liver BFU-Es increase (Migliaccio et al., 1986, J. Clin. Invest., 78: 51-60). Further descriptive analyses suggest early thymus colonization at week 7 (Haynes et al., 1988, J. Exp. Med., 168: 1061-1080) and bone marrrow colonization at weeks 10-11 (Charbord et al., 1996, Blood, 87: 4109-4119). By 15-20 weeks in gestation it is thought that both the bone marrow and fetal liver contain cells characteristically described as hematopoietic stem cells and such early fetal liver cells have been used successfully in clinical transplantations. Intrabody regions been examined for adult-type hematopoietic progentors/stem cells.

Clonogenic myeloid progentiors have been found in the yolk sac and body of 25 to 50 day gestational stage human embryos (Huyhn et al., 1995, Blood, 86: 4474-4485). Erythroid and multipotent progenitors are also present in these tissues, and between 30 and 40 days into gestation, as many non-erythroid progenitors are found in the eviscerated embryo as in the liver. Descriptive immunhistochemical analyses have localized a cluster of cells adhering to the ventral endothethial wall of the dorsal aorta in 5-week preumbilical human embryos. These are phenotypically identical

(CD34^{+},CD38^{-}) to adult hematopoietic progenitor/stem cells (Tavian et al., 1996, <u>Blood</u>, 87: 67-72). The presence f these cells corr sponds well to findings in avian (Cormier and Diet rlen-Li vre, 1988, <u>Development</u>, 102: 279-285) and murine (Garcia-Porrero et al., 1995, <u>Anat. Embryol (Berl.)</u>, 192: 425-435; Medvinsky et al., 1996, <u>Blood</u>, 87: 557-566) embryos at equivalent developmental stages where immatur hematopoietic progenitors/stem cells are found on ventral wall on the dorsal aorta. Finally CD34^{+} cells from the human intraembryonic region yield large multilineage colonies after coculture on bone marrow stromal cells. Thus, like avian, amphibian and murine embryos, the human embryo has potent hematopoietic activity within the intraembryo region containing the dorsal aorta.

<u>Analysis of HSC proliferation and differentiation according to the invention</u>

HSC expansion and differentiation is determined as follows: For CFU-S assay the recipient mice are sacrificed on day 11 post-transplantation, their spleens are removed and fixed in Bouin's solution (see Humason, G.L., 1979, <u>Animal Tissue Techniques, 4th Edition</u>, W.H. Freeman and Company, San Francisco). Spleen colonies are counted under a dissecting microscope. For the long-term repopulating activity (HSC) assay, the peripheral blood of recipient mice is analysed 2 times; once at 1.5 to 2.5 months and then at 5.5 to 8 months post-transplantation. Blood (100-200 μl) is collected from the tail vein of the mice and digested with proteinase K, followed by phenol-chloroform extraction and isopropanol precipitation as previously described (Müller et al., 1994, supra). Donor male cell contribution is assessed semi-quantitatively by PCR using YMT2/B primers and myogenin gene specific oligos as the DNA normalization control (Müller and Dzierzak, 1993, <u>Development</u>, 118: 1343-1351). The myogenin specific oligos used are:

MYO-1   TTA CGT CCA TCG TGG ACA GC   [SEQ ID NO: 3]
MYO-2   TGG GCT GGG TGT TAG TCT TA   [SEQ ID NO: 4]

The reactions for the combination of YMT2/B and myogenin oligos are initial heating at 95°C for 5 minutes, followed

by 30 cycles at 94°C for 10 seconds, 60°C for 30 seconds and 72°C for 35 seconds and a final single cycle at 37°C for 10 minutes. The cycles are performed in any of a number of commercially available thermocyclers, e.g. a Techne PHC-2 thermocycler. The sizes of the amplified products are 342 bp (YMT2/B) and 245 bp (myogenin). The products are separated on 2.0% agarose gels and transferred to nylon membranes followed by hybridization with ^{32}P-labeled YMT2/B and myogenin (MYO) specific probes. The percentage of engraftment is determined by quantitation of radioactive fragments on a phosphorimager and plotting on a graph derived from data obtained in the same PCR of serial dilutions of male DNA (Müller et al., 1994, supra).

i. Multilineage analysis of donor cell contribution

To test the contribution of donor HSCs from day 10 p.c. cultured AGM in the different lympho/hematopoietic lineages in long-term transplanted mice, positive animals are sacrificed and analysed. The following tissues and cells are analysed; peripheral blood, bone marrow, thymus, spleen, lymph nodes, B-cells, macrophages and mast cells (Müller et al., 1994, supra). Briefly, B-cells from spleen cell suspensions (non-adherent fraction) are enriched in culture by stimulation with 10 mg/ml lipopolysaccharide (Sigma) for 3 days; macrophages are initially enriched as an adherent cell fraction from the peritoneum and spleen, followed by expansion in culture with 10% L929-conditioned medium (source of M-CSF) for 4-10 days; mast cells are cultured for 5 weeks in 10% WEHI-3 conditioned medium. Enrichment of B cells is determined by antibody staining specific for B220 and FACS analysis; in a representative experiment, 97.5% enrichment was observed. Macrophages are stained directly in culture with Mac-1 antibody; 100% of cells are found to be positive for this marker. Mast cells are stained with IgE antibody (Clone SPE-7, Sigma) followed by a secondary PE conjugated goat anti-mouse antibody (Southern Biotechnology Associates, Inc.); in a sample experiment, 53-81% of cells were found to be positive by FACS analysis. These antibodies are, therefore, efficacious in binding these

several cell types. Ficoll gradient fractionation is
performed to remove dead cells from the B cell cultures.

ii.   C 11 preparation, fluorescence surfac staining and
flow cytometry

Embryos of the desired developmental stage (the day of
appearance of the vaginal plug in mated females is
designated as day 0) are removed and embryonic tissues (AGM
region and liver) dissected as previously described (Müller
et al., 1994, supra). Cell suspensions are obtained by
incubation of embryonic tissues at 37°C for 1 h in 0.04%
collagenase (Sigma) in L-15 medium (Flow Lab) supplemented
with fetal calf serum (5-10%) followed by gentle mechanical
dispersion. Viable cells are counted using trypan blue
exclusion.

Cell sorting techniques are well known to those skilled
in the art and are described in Sprangrude et al., 1988
(Science, 241:   58-62). The antibodies used in flow
cytometric sorting and analysis may be prepared using
standard methods or may be obtained commercially; those
discussed in the below are all commercially available from
Pharmingen, San Diego, CA, except for AA4.1. The monoclonal
antibodies (mAbs) used are direct conjugates with either
FITC (fluorescein isothyocyanate), PE (phycoerythrin) or
biotin. Single cell suspensions from AGM and liver are
prepared as indicated previously (Miles et al., 1996, J.
Neuroimmunol., 1:   19-24). Cells are suspended in L-15
medium with 5% FCS. Incubation with CD16/CD32 mAb is
performed for 20 minutes on ice to lower non-specific
staining, followed by incubation with specific mAbs for 20-
30 minutes on ice. Cells are washed twice and incubated
with labeled streptavidin, such as PE-conjugated
streptavidin (e.g. as supplied by Pharmingen) or Red-670
streptavidin (e.g. that supplied by Gibco BRL) when
required. Labeled cells are finally washed twice in L-15
medium, resuspended in the same medium containing 0.5 mg/ml
propidium iodide (PI, Sigma) and filtered through a nylon
mesh screen to remove debris. Antibodies of use according

to the invention include, but are not limited to the following: FITC anti-c-kit (3C1), PE anti-CD2 (RM2-5), PE anti-CD4 (RM4-5), PE anti-CD8 (53-6.7), PE anti-Mac-1 (M1/70), PE anti-CD16/32 (2.462), PE anti-CD44 (IM7), PE anti-B220 (RA3-6B2), PE anti-Gr-1 (RB6-8C5), PE anti-Thy 1.2 (53-2.1), PE anti-Sca-1 (E13-161.7), PE anti-Ter119, biotinylated anti-CD34 (RAM34) and biotinylated AA4.1. Viable cells are defined by exclusion of PI-positive and high obtuse scatter or low forward scatter on a dual-laser FACStar Plus or Vantage cell sorter (Becton Dickinson, San Jose, CA). Analyses are performed on a fluorescent cell sorter. Sorted cells are collected and counted and, when possible, re-analyzed for purity (>90%). Control cells may be stained with any of a number of labeled primary antibodies; in the experiments described below, biotinylated rat $IgG_{2a/a}$ was employed, followed by streptavidin-PE, PE-conjugated IgG_{2b} or FITC-conjugated IgG_{2b} (also available from Pharmingen).

For lineage reconstitution analysis cell suspensions are prepared from femoral bone marrow or spleen and stained with B220 or Mac-1.

iii. Reconstitution analysis.

For transplantation of sorted embryonic cells, 2 to 3 month old female mice are used as recipients. Mice are irradiated with a split dose of 1000 rads from a ^{60}Co source as previously published (Müller et al., 1994, supra). After irradiation, mice are maintained on antibiotic water containing 0.16% neomycin sulfate (Sigma) for 4 weeks. Cells are injected intravenously (0.5 ml per mouse) in the tail vein of irradiated recipients. Transplanted animals are bled from the tail vein at 1-2 months and 5-8 months post-transplantation to monitor reconstitution. PCR analysis is performed on peripheral blood DNA for the presence of the donor-specific marker gene. In the case of the experiments described below, the *LacZ* gene was employed; however, any molecular marker not present in recipient cells may be used. An endogenous marker, such as a housekeeping gene against which expression of the donor may be quantified, is also

amplified.

Genomic DNA is isolat d from peripheral blood and tissues of recipient animals as previously described (Müller et al., 1994 supra). DNA samples (200 ng) are analyzed by PCR using the following oligonucleotide primers: for myogenin-specific sequences (Myo1) TTACGTCCATCGTGGACAGC [SEQ ID NO: 3] and (Myo2) TGGGCTGGGTGTTAGTCTTA [SEQ ID NO: 4]; and for LacZ specific sequences (lacZ1) GCGACTTCCAGTTCAACATC [SEQ ID NO: 5] and (lacZ2) GATGAGTTTGGACAAACCAC [SEQ ID NO: 6]. DNA is subjected to an initial 5 minute denaturation at 94°C followed by 30 cycles of denaturation (5 seconds at 94°C), annealing (30 seconds at 60°C) and elongation (30 seconds at 72°C). Serial dilutions of blood DNA from a transgenic animal are used as a PCR control to evaluate the levels of donor cell reconstitution in transplanted mice. The sizes of the amplified LacZ and myogenin PCR products are 670 bp and 245 respectively. The products are separated on 1.5-2% agarose gels, transferred to nylon membranes and hybridized with myogenin and Lac-Z probes.

For reconstitution analysis genomic DNA is isolated from bone marrow, spleen, lymph nodes, blood and thymus. B cells are either enriched by sorting B220$^+$ cells from bone marrow or spleen or by a spleen cell suspension culture stimulated with 10 mg/ml lipopolysaccharide (Sigma) for 7 days. Macrophages are either enriched for Mac-1$^+$ cells from bone marrow or spleen or as an adherent cell fraction from the peritoneum followed by expansion in culture with 10% L929 conditioned medium for 4-10 days.

Preparation of a chimeric organ culture

AGM tissues are isolated from E11 mouse embryos, e.g. from (CBAxB10)F1 mice, and cultured as described above except that the explanted tissues are irradiated with 0, 500, 750 or 1000 rads of gama irradiation. It has been found that endogenous HSC activity is completely eliminated with a dose of 500 rads. Cultured AGM regions are injected with 0.5-1 microliters of a cell suspension containing 200,000-400,000 transgene-marked exogenous cells; in the experiments described below in Example 4, human β-globin-marked cells

(line 72) are employed. After 3-4 days of culture tissues
are harvested and single cell suspensions injected into
l thally-irradiated adult recipients to test for HSC
activity.   Donor cell contribution is m asured by PCR
analysis of peripheral blood DNA (human β-globin marker) at
1 and 4 months post-transplantation, as described above.

EXAMPLE 1

a.   Induction and expansion of CFU-S in cultured, isolated
embryonic tissues

Using an experimental approach whereby isolated embryonic
tissues can be cultured separately from other tissues so
that cells cannot migrate or circulate between hematopoietic
sites, the AGM region, YS and liver were examined for the
generation of definitive CFU-S progenitors.   As shown in
Figure 1, the culture system was established with whole
embryonic tissues cultured on semi-permeable filters at the
air/medium (or "air/liquid") interface for 2-3 days followed
by dispersion into a single cell suspension and injection
into lethally irradiated mice.

When day 9 p.c. tissues from 13 embryos were individually
cultured and cell suspensions injected, no CFU-S were found
11 days post-transplantation in the recipient mice.
However, beginning at early and mid-day 10 p.c. significant
CFU-S activity could be found in cultured AGM and YS but not
in liver, control head/heart or body remnants (Table 1).   At
the 32-33 somite pair stage, the number of CFU-S per
cultured AGM was found to be 4.8 as compared to 0.5 CFU-S
per cultured YS, representing over a 9 fold difference in
progenitor number between the tissues.   At the 34-35 somite
pair stage the number of CFU-S in cultured AGM regions (9.3
CFU-S/tissue) again surpassed the number in cultured YS (1.0
CFU-S/tissue) by a factor of 9.   When day 11 p.c. embryo (42
somite pair) tissues were examined, large numbers of CFU-S
were found in the AGM region as compared to the YS and
liver.   The AGM region, YS and liver from five mid-day 11
p.c. (CBA x C57Bl/10) F1 embryos were cultured separately
for 3 days.   Each group of tissues was pooled and
transplanted into five irradiated (CBA x C57Bl/10) F1 adult

recipients, one embryo equivalent of tissue per recipient. As shown in Figure 2 the resultant spleen colonies from one representative experiment were 3 fold more abundant per cultured AGM region (31.4 CFU-S/tissue) than cultured YS (9.2 CFU-S/tissue) and 15 fold more per cultured AGM than per cultured liver (1.8 CFU-S/tissue). The weights of the spleens of the various recipient groups differ dramatically and correspond directly with the number of CFU-S produced. No colonies were observed in the control non-injected recipients. Finally at day 12 p.c., large numbers of CFU-S are also found in cultured liver (0.2 embryo liver equivalents produced confluent spleen colony growth, data not shown).

To determine whether the culture system provides a normal supportive environment for CFU-S progenitors, CFU-S numbers from cultured tissues were compared to directly transplanted tissues (Medvinsky et al., 1993, supra). The cumulativ results from numerous experiments at early and mid-day 10 p.c. (32-33, 34-35 and 36-38 somite pair) and day 11 p.c. (42 and 46 somite pair) are presented in Figure 3. Data were obtained from 1-6 culture experiments performed with four 32-33, ten 34-35, eighteen 36-38, two 42 and four 46 somite pair embryos; the CFU-S results of directly transplanted uncultured AGM, YS and liver from day 10 p.c. (34-35 and 36 -38 somite pair) and day 11 p.c. (46 somite pair) embryos (Medvinsky et al., 1993, supra) are shown. CFU-S experiments with directed transplanted uncultured 32-33 and 42 somite pairs were not performed. For the 36-38 somite pair embryos, no cultures of yolk sac or liver were established. The X-axis indicates the age and somite number of the embryos used for explantation to culture and for direct analysis of uncultured tissues. The Y-axis indicates the number of CFU-S per tissue. At day 10 p.c., uncultured AGM region harbors 0.9-1.2 CFU-S progenitors (Medvinsky et al., 1993, supra). However, cultured AGM regions from 32-33, 34-35 and 36-38 somite pair stage embryos yielded 4.8, 6.2 and 9.6 CFU-S/tissue, respectively. Thus, within the cultured AGM region, CFU-S numbers are increased 4-9

fold over the numbers of CFU-S per uncultur d, directly transplanted AGM. While capable of maintaining CFU-S progenitors, no comparable increases in CFU-S numbers were found in cultured day 10 p.c. YS. At day 11 p.c., CFU-S numbers in the AGM region were again increased (6-fold) during the culture period while cultured YS and liver show only small increases over uncultured tissues. These data demonstrate that beginning at day 10 p.c., the CFU-S progenitor pool is initiated and expanded autonomously in cultured AGM tissue and to a greater potency than in isolated YS and liver and are consistent with the idea that the AGM region is a major generator of CFU-S activity.

b. Competence of cultured AGM tissue to generate and expand HSCs autonomously

The successful maintenance and growth of CFU-S progenitors in cultures led to experiments testing for definitive adult HSC activity. The in vivo long term reconstitution of lethally irradiated mouse recipients is the only assay at present able to reveal unequivocally HSCs. As described above and shown in Figure 1, tissues from male embryos were cultured for 2-3 days and cells injected into lethally irradiated female recipients. At 2 and 8 months post-transplantation, recipient peripheral blood DNA was tested for the presence of the donor male marker using myogenin gene specific oligos as the DNA normalization control (Müller and Dzierzak, 1993, supra; Medvinsky et al., 1993, supra). Southern blot analysis was performed after gel electrophoresis and hybridization with ^{32}P-labeled probes for the YMT2/B and myogenin (MYO) specific fragments. An autoradiogram is shown. Controls for donor cell contribution include dilutions of male DNA into female DNA and are as indicated; 100%, 10% and 1%. The percentage contribution in each of the peripheral blood samples is indicated at the bottom of each lane. Note that only strong YMT2/B signal is observed in mice transplanted with cultured AGM region. In the representative experiment shown in Figure 4A, the YMT/2B male marker was detected to high levels (greater than 10%) in 2 out of 2 recipients receiving

cultured day 10 p.c. AGM but not in any of the 2 and 3 recipients receiving cultured YS or cultured liver respectively. The cumulative results of 13 experiments (Table 2) demonstrate that at day 10 in gestation, only cultured AGM can repopulate lethally irradiated recipients to high levels (greater than 10%). Moreover, the efficiency of high level donor cell repopulation of recipients from cultured 35-38 somite pairs AGM was greatly increased (89%; 24 positive out of 27 transplanted with about 2 embryo equivalents) when compared to the direct transplantation (3%; 3 positive out of 96 transplanted with 1.2 embryo equivalents) of AGM region cells of this stage (Müller et al., 1994, supra). Thus, in correspondence with autonomous generation and increases in number of CFU-S progenitors in cultured AGM, HSCs are also autonomously generated and greatly increased (approximately 15 fold, taking into account the embryo equivalents transplanted) within the isolated day 10 p.c. AGM region during culture.

While levels of donor cell engraftment greater than 10% generally represent full multilineage repopulation, numerous transplant recipients of cultured day 10 p.c. AGM were tested for whether they contained the donor derived marker in all hematopoietic tissues and the lymphoid and myeloid lineages at greater than 8 months post-transplantation. The following tissues were dissected and cell lineages isolated: BL, peripheral blood; BM, bone marrow; T, thymus; S, spleen; LN, lymph nodes; S-M, splenic macrophages; S-B, splenic B cells; P-M, peritoneal macrophages. DNA was examined for the presence of the Y chromosome marker by quantitation of specific hybridization of YMT/2B and myogenin PCR products after Southern blot transfer. As shown in Figure 4B for four representative recipients, it was found that all tissues and the T and B lymphocyte and myeloid lineages were repopulated to 100% with donor-derived cells. Also, cultured bone marrow macrophages and mast cells were found to be 100% donor-derived (not shown). Tissues (thymus, spleen and bone marrow) from 5 negative recipients injected with day 10 p.c. cultured YS were also

analysed and no contribution of donor cells was found (data not shown). Thus, the culture conditions do not affect the high level multilineage potency of HSCs arising from cultured day 10 p.c AGM and yield identical results to those previously reported with directly transplanted day 10 p.c. AGM region cells (Müller et al., 1994, supra).

HSC potential at the day 11 p.c. stage of development was examined by culture of YS, liver, body remnants and AGM region. A limiting dilution experiment revealed high level HSC activity in cultured YS and AGM but not in liver or body remnants (Figure 5). At greater than 6 months post transplantation, recipients receiving day 11 p.c. cultured AGM region, YS, liver and body remnant cells were analysed for donor cell contribution by peripheral blood DNA PCR specific for YMT/2B and myogenin. Either 0.25 embryo equivalents (ee) or 0.08 ee of cultured tissues were injected into female recipients. Controls for donor cell contribution are mixes of male and female DNA and are indicated as 100%, 10%, 1% and 0.1%. Percentage contribution of donor-derived cells was determined by phosporimaging and is indicated for individual recipients at the bottom of each lane. While 0.25 embryo equivalents of YS and AGM resulted in high level (greater than 10%) repopulation, only the AGM region was able to give some repopulation with 3 fold fewer cells (0.08 embryo equivalents) and no repopulation was observed when cultured liver or body remnants (Body R) were transplanted, indicating a difference in the number of HSCs and/or the potency of the HSCs between these tissues.

Finally to determine the earliest stage at which HSC activity can be generated, day 9 p.c. embryonic tissues was cultured. In two experiments with tissues from a total of 18 embryos no repopulating activity was found in cultured AGM, liver or YS. Taken together, these results demonstrate that HSC activity is autonomously initiated and expanded in the AGM region but not in the YS or liver beginning at day 10 p.c. At least from day 10 p.c. onwards, the generation and expansion of the HSCs in the AGM region is not dependent

upon migration of cells from the YS.   Also, this initial
increase of definitive adult HSC activity in the AGM region
is not dependent on the liver microenvironment.

c.   Localization of the generation and expansion of HSCs
within the AGM

At day 10 p.c. the mesodermal region that forms the AGM is
composed of the dorsal aorta, the pronephros, mesonephros
and the genital ridge. At later stages of development, the
metanephros (the primordium for the adult kidneys) and the
gonads form here (Kaufman, 1992, The atlas of mouse
development., Academic Press, London, UK.   pp. 465-468).
The data obtained from amphibian and fish models suggest
posterior to anterior migration of hematopoietic cells
(Turpen and Knudsen, 1982, supra; Detrich et al., 1995,
Proc.   Natl.   Acad.   Sci.   U.S.A.,   92:   10713-10717).
Therefore, it was of interest to localize along the
anterior-posterior axis which region within the AGM is
responsible for the LTR-HSC generation.

The anterior and posterior sections of the AGM region were
separated after dissection from day 10 p.c. male embryos
(Figure 6A).   The schematic drawing indicates the tissue
comprising this region: pronephros, mesonephros, gonads
and dorsal aorta.   The anterior AGM region contains the
pronephros, parts of the mesonephros, dorsal aorta and
genital ridge.   The posterior section contains the other
parts of the mesonephros, dorsal aorta and genital ridge.
These sections were cultured for 2 days and then tested for
HSC activity in lethally irradiated female mouse recipients.
The level of donor-derived reconstitution was determined by
Y chromosome specific PCR analysis of peripheral blood DNA
(Figure 6B).   Southern blot analysis of PCR-amplified
peripheral blood DNA from recipients receiving cultured AGMa
(anterior), AGMp (posterior), YS or liver was performed.
Controls include mixes of male and female DNA as indicated;
100%, 10% and 1% and percentage contribution of male donor
cells in individual female recipients is shown for each
lane.   In two separate experiments at greater than 4 months
post-transplantation, the cultured anterior section

repopulated all recipients (five out of five recipients) to
a much higher percentage (10-100%) than the cultured
posterior section.   No recipients transplanted with
posterior AGM region cells were found to be repopulated to
greater than 5% and as found in previous experiments,
cultured intact YS and liver showed no repopulating
activity.   Additional experiments were performed to
determine whether CFU-S present in the AGM region would co-
localise with HSC activity.   When a mixture of day 10 p.c.
AGM regions from 14 embryos (33-38 somite pairs) were
cultivated as described above, no difference in CFU-S number
was found between the anterior (2.8 CFU-S/tissue) and
posterior (2.4 CFU-S/tissue) regions.   In a second
experiment with 5 late day 10 p.c. embryos (38-40 somite
pairs), 12.6 CFU-S per anterior AGM and 8.6 CFU-S per
posterior AGM region were found.   Thus, there is no strict
correlation in the spatial development of CFU-S progenitors
and HSCs.   These results demonstrate that the anterior
section of the day 10 p.c. AGM region which consists of the
pro/mesonephros and part of the dorsal aorta and genital
ridge is most important for the autonomous generation and
expansion of definitive HSC activity.

Despite a long history of studies on developmental
hematopoiesis, the embryonic origin of the definitive
hematopoietic system in adult mammals was unknown. For over
twenty years it has been widely accepted that the YS, in
which the first differentiated hematopoietic cells can be
detected, is the source of the founder hematopoietic stem
cells for the fetal liver and subsequently the adult bone
marrow (Moore and Metcalf, 1970, supra). However, studies
using avian (Dieterlen-Lievre, 1975, supra; Lassila et al.,
1978, supra) and amphibian (Turpen et al., 1981, supra;
Turpen and Knudsen., 1982, supra; Kau et al., 1983, supra;
Maeno, et al., 1985, supra) embryos have demonstrated that
an independent source of hematopoietic activity functions
within the embryo proper.   In these species, it has been
clearly shown that YS-derived hematopoiesis is transitory
(possibly providing for the immediate needs of the embryo)

and does not supply the adult with full hematopoietic potential. Hence, the hematopoietic hierarchy of the YS is limited in its complexity and is incomplete compared to that in the definitive hematopoietic territories (Dzierzak and Medvinsky, 1995, supra). In the mouse this is consistent with the failure of numerous research groups to uncover definitive cells such as CFU-S progenitors and HSCs within the early YS (Sonoda et al., 1983, supra; Perah and Feldman, 1977, supra; Symann et al., 1978, supra; Samoylina et al., 1990, supra; Medvinsky et al., 1993, supra; Müller et al., 1994, supra; Harrison et al., 1979, Blood, 54: 1152-1157). An alternative site of potent hematopoietic activity has been identified in the mouse embryo (Medvinsky et al., 1993, supra; Godin et al., 1993, Nature, 364: 67-70) which is the anatomical analogue of the intrabody definitive hematopoietic source in avian and amphibian embryos. The AGM region, which at earlier stages of development represents intra-embryonic splanchnopleura, is a strong alternative candidate for the origin of definitive hematopoiesis in the mammalian embryo. Temporal analysis of lympho/hematopoietic progenitors at day 8 p.c. (Godin et al., 1995, Proc. Natl. Acad. Sci. USA, 92: 773-777) and definitive CFU-S progenitors (Medvinsky et al., 1993, supra) and HSCs (Müller et al., 1994, supra) at day 10 p.c. strongly suggest their temporal appearance more or less in parallel in both the AGM region and the YS of the mouse embryo. However, these studies neither reveal the primary embryonic source of these cells nor disclose the relationship between the embryonic hematopoietic tissues which may actively exchange cells through the circulation or interstitial migration.

In the present invention a culture approach which prevents possible cellular interchange between the individual hematopoietic tissues of the embryo and preserves the potentially important cellular microenvironment of the explant has been developed. It has been shown in other developmental systems that in vitro culture of embryonic rudiments can be used for ontogenic investigations

(Kinoshita and Asashima, 1995, <u>Development</u>, 121: 1581-1589); J nkinson et al., 1982, <u>Eur. J. Immunol.</u>, 12: 583-587; Kratochwil et al., 1983, "Embryonic induction." In: <u>Cell interactions and Development: Molecular mechanisms.</u>, ed. K.M. Yamada, pp. 99-122, New York: J. Wiley and Sons Press; Tickle and Eichele, 1994, Annual Rev. Cell Biol. 10, 121-152; Serra and Moses, 1995, <u>Development</u>, 121: 3057-3066). The *in vitro* culture described enables reproducible discrimination between the YS and AGM region for the initiation and expansion of definitive hematopoietic stem cells and progenitors.

It is demonstrated that CFU-S progenitors can be maintained during the 2-3 day culture of whole, intact AGM, YS and liver from day 10, 11 or 12 p.c. embryos. However, only the cultured AGM region can significantly increase the number of CFU-S progenitors within it during the culture period. In contrast to the normal embryo, it appears that CFU-S generated within the isolated, cultured AGM region ar unable to emigrate and disseminate throughout the embryo (Medvinsky et al., 1996, supra). Thus, they accumulate *in situ* and surpass the numbers that can be observed in uncultured AGM region (Medvinsky et al., 1993, supra). Also, the significant delay of onset and the low numbers of CFU-S detected in the isolated, cultured liver and YS strongly suggest colonization of these tissues by AGM generated CFU-S progenitors.

Of greater significance in the establishment of the definitive hematopoietic system and in strong support of th CFU-S findings are the results of the long term *in vivo* transplantations of cells from culture. It is shown that at day 10 p.c., only the AGM region is able to initiate and expand definitive adult HSC activity. In general, uncultured day 10 p.c. AGM cells cannot reliably repopulate adult recipients. It was previously found that in only 3 out of 96 recipients were donor cell positive with directly transplanted AGM region cells (Müller et al., 1994, supra). Here it is shown that day 10 p.c. AGM region in culture can expand HSCs to numbers that can consistently provide

complete hematopoiesis to the adult (in 24 out of 27
recipients) while cultured YS failed to yield HSC activity
until day 11 p.c. and at a lower frequency than cultured
AGM. Taken together these data provide strong evidence that
the AGM region is the first (and possibly the only) tissue
initiating development of HSCs at the pre-liver stage of
hematopoiesis. It seems likely that HSCs appearing in the
YS and in other tissues of the embryo at day 11 p.c. is the
result of dissemination of HSCs from the AGM region. This
would be much like that observed in avian embryos where
heterospecific chimeras between quail embryo and chick
blastoderm revealed quail cells in the YS blood islands
shortly after grafting (Martin et al., 1978, _Cell
Differentiation_, 7: 115-130).

Orthotopic embryo grafting experiments in avian and
amphibian species clearly demonstrate two separate
mesodermal sites of hematopoietic development in the embryo
and thus evolutionary conservation between species would
favour the birth of pre-stem cells _in situ_ in the mouse AGM
region. At the moment the possibility that the culture
conditions used are incompatible for HSC initiation in th
YS cannot be ruled out. However, this seems unlikely since
it would imply that the early mouse embryo employs
simultaneously, two very distinct mechanisms for the
generation of definitive hematopoiesis in the two different
sites. Another more realistic possibility implies that
there may exist within the conceptus, at an undetermined
site, a pre-HSC with multilineage potential which for some
reason (lack of homing receptors, low proliferative
potential in an adult microenvironment, etc.) cannot
repopulate adult recipients to high levels. Numerous events
such as a progressive increase in expansion potential and/or
maturation to an adult phenotype may occur in the AGM region
to generate high level HSC activity. This would be
consistent with previous studies demonstrating limited
and/or low level repopulation with YS cells (Sonoda et al.,
1983, supra; Harrison et al., 1979, supra; Weissman et
al., 1978, "Fetal hematopoietic origins of the adult

hematopolymphoid system." In: Differentiation of Normal
and Neoplastic Hematopoietic Cells , Cold Spring Harbor
Laboratory Press, Cold Spring Harbor, New York, pp. 33-47;
Toles et al., 1989, Proc. Natl. Acad. Sci. USA, 86:
7456-7459) and differentiated ES cells (Müller et al., 1994,
supra) when transplanted into an embryonic and neonatal
environment, respectively, and the presence of multilineage
progenitors in the para-aortic splanchnopleura (Godin et
al., 1993, supra). Recent in vitro work on a multipotential
AA4.1 positive cell from the mouse splanchnopleura suggests
that this may be a candidate pre-stem cell (Godin et al.,
1995, supra). Also, it is a formal possibility that
primordial germ cells (PGCs) may play a role in the
generation of HSCs. The colocalization of PGCs with CFU-S
in the embryo body supports this notion (Medvinsky et al.,
1993, supra; Medvinsky et al., 1996, supra), as does the
finding that PGCs retain plasticity to form ES cells (Matsui
et al., 1992, Cell, 70: 841-847) and possibly hematopoietic
progenitors in vitro (Rich, 1995, Blood, 86: 463). It is
interesting that the most potent initiation of HSCs in the
culture system is localized to the anterior AGM region.
This may suggest a posterior to anterior gradient of
activity and/or migration of cells in the mouse, possibly to
the pronephros (the most anterior structure of the embryonic
kidney) which has been shown to play an important role in
the development of the definitive hematopoietic system in
amphibians (Turpen et al., 1981, supra; Turpen and Knudsen,
1982, supra; Kau et al., 1983, supra; Maeno et al., 1985,
supra).

It has been generally thought that the maturation of HSC
activity in the embryo occurs within the fetal liver since
increasing stem cell activity has been found there from day
11 p.c. onwards (Müller et al., 1994, supra; Fleischman et
al., 1982, Cell, 30: 351-359; Morrison et al., 1995,
Proc. Natl. Acad. Sci. USA, 92: 10302-10306; Jordan, et
al., 1990, Cell, 61: 953-963). However, the high level of
HSC activity which develops in cultured day 10 p.c. AGM
suggests that each individual AGM region autonomously is

able to produce a pool of HSCs sufficient to provide the adult organism with HSCs throughout its life in the absence of the fetal liver microenvironment. Subsequent to this burst of activity in the AGM region, HSC expansion in the liver (1-2 days later) overtakes the AGM region, thus suggesting that colonization of the fetal liver by AGM HSCs is followed by further expansion and maturation steps. The significance of such excessive secondary expansion of HSCs in the fetal liver is not clear, taking into account the oligoclonality of adult hematopoiesis (Jordan et al., 1990, supra; Jordan et al., 1990a, Genes Dev., 4: 220-232).

As at least a 15-fold enhancement in HSC activity from cultured day 10 p.c. AGM region compared to uncultured AGM has been observed, this is the first demonstration of expansion of HSC activity in vitro. There are two means by which HSC numbers increase in the AGM region during the culture period: 1.) by maturation of a pool of pre-stem cells to definitive HSCs or 2.) by clonal expansion of a single or few starting HSCs. Whatever the mechanism, in contrast with Dexter-type long term bone marrow cultures in which HSCs are maintained but not expanded (Harrison et al., 1987, Blood, 69: 1021-1025), the unique temporal and spatial properties of the embryonic AGM region should lead to a better understanding of the inducing and/or expansion factors necessary for the initiation of the definitive adult hematopoietic system in mammals. The individual cellular components and molecular mechanisms by which the embryo attains HSC expansion provide useful applications for gene therapy. The invention also is useful for induction of pre-HSCs to HSCs.

Finally, the in vitro cultures offer an advantageous test system for identifying specific developmental hematopoietic defects in numerous recently reported homologous recombination knock out mice (Pandolfi et al., 1995, Nature Genetics, 11: 40-44; Tsai et al., 1994, Nature, 371: 221-226; Okuda et al., 1996, Cell, 84: 321-330; Scott et al., 1994, Science, 265: 1573-1577). Such homozygous mutant mice die between day 11-16 p.c. and show a lack of

definitive hematopoiesis. Analysis of YS, AGM and liver
from mutant day 10-11 p.c. embryos in the culture system
described here should indicate whether defici ncies occur in
the pre-stem cell, stem cell, progenit r cell or
microenvironmental compartments.

<div align="center">EXAMPLE 2</div>

Preparation of an enriched _in vitro_ culture of inducible
and/or expandable HSCs

In order to obtain an enriched culture of HSCs it is
possible to use standard cell separation techniques. Such
standard techniques are described below as well as HSC
markers, in order to determine whether an enriched culture
of HSCs is obtained according to the invention.

a. Establishment of an HSC cell surface marker in day 11
p.c. AGM and liver

To determine the cell surface phenotype of the first HSCs
within the developing mouse embryo, cells from the AGM
region and early fetal liver were examined for expression of
the c-kit cell surface marker. For the generation of
embryos, (CBA x C57BL/10) F1 females were bred with Ly-6E.1
LacZ transgenic homozygous males (Miles et al., 1996,
supra). Cells were obtained from pooled AGM regions and
livers of day 11 p.c. embryos and stained with c-kit
specific antibody or isotype-matched control. As shown in
Figure 7A and C, three sorting regions were set according to
the relative levels of c-kit surface expression: c-kit$^+$(R1),
c-kit^{lo}(R2) and c-kit$^-$(R3). The c-kit$^+$ populations of both
the AGM region and liver appear to contain cells mainly with
high forward scatter indicating that they are large in size.
Side scatter analysis shows that all populations of AGM
cells are highly granular (with the c-kit$^+$ population most
granular) when compared to liver. Varying numbers of cells
from each of the c-kit sorted regions were transferred into
unmarked irradiated recipient mice and donor cell
engraftment measured at one month and greater than six
months post-transplantation. Recipient peripheral blood
DNA was analysed by PCR for the presence of the donor LacZ
transgene. As shown for one representative experiment in

Figure 7B and D, blood DNA samples from animals receiving c-kit^{lo} and c-kit$^-$ cells from AGM or liver were negative (<1%) for donor Lac-Z signal. Only when c-kit$^+$ cells were transplanted did recipients exhibit high level donor cell repopulation. The cumulative results from several experiments (shown in Table 1) demonstrate that high-level reconstituting activity resides only in the c-kit$^+$ population. A mean of $6.6 \pm 4.5 \times 10^3$ c-kit$^+$ cells from AGM yielded 12 out of 20 reconstituted recipients. However, 10-fold more cells or a mean of $63 \pm 32 \times 10^3$ c-kit$^+$ liver cells are needed to get similar reconstitution in 7 out of 13 recipients.

To determine whether this high level repopulation was multilineage, 14 recipients that received AGM c-kit$^+$ cells and 13 recipients that received liver c-kit$^+$ cells were examined. Hematopoietic tissues (blood, thymus, spleen, lymph nodes and bone marrow) and purified B cells and macrophages were assayed by PCR analysis for the presence of the Lac-Z transgene. As shown in Table 2, all 14 AGM recipients maintained the same level of repopulation in peripheral blood after 6-8 month. In 6 out of 12 animals that showed 100% donor derived peripheral blood, all the hematopoietic tissues, B cells and macrophages were 100% positive. The other animals showed some small variation in the level of repopulation in the individual tissues and lineages. Recipients reconstituted with liver c-kit$^+$ cells did not show the same stability in repopulation as the AGM recipients. Three out of nine recipients decreased peripheral blood signal from 100% to 10%. Two recipients with an initial 10% reconstitution, completely lost donor signal in blood after 6 months, although one is 1 to 10% positive in bone marrow and macrophages and the other is 1 to 10% positive in B cells and macrophages. These data show that all HSCs in the AGM and liver are within the c-kit$^+$ fraction, that these cells can yield complete multilineage repopulation and that the reconstitution potential of the c-kit$^+$ liver population is more restricted than that of the AGM.

b.   Temporal analysis of HSC potential of c-kit⁺ cells.

Since the hematopoietic system of the early- and mid-
gestation embryo is rapidly expanding and differ ntiating,
c-kit expression was analysed in AGM and liver cell
suspensions from day 10, 11 and 12 p.c. embryos. As shown
in Figure 8, increases in the percentage of c-kit⁺ cells are
found at day 11 p.c. in both AGM and liver when compared to
day 10 p.c. However, at day 12 p.c., the percentages
decrease in both tissues but remain higher than at day 10
p.c. To determine whether these changes were reflected in
the functional characteristics of these c-kit⁺ populations,
reconstitution experiments were performed (Table 3).
Peripheral blood DNA of each of the recipients was examined
by PCR for the presence of the donor Lac-Z transgene. When
considering the number of embryo equivalents, th
reconstitution potential of liver c-kit⁺ cells at day 11
p.c. is similar to the AGM c-kit⁺ cells, although 10 times
more liver c-kit⁺ cells are required. At day 12 p.c., the
liver c-kit⁺ population appears to contain more abundant HSC
activity than the AGM region. As expected from the limited
number of transplant recipients, no repopulated recipients
were observed with day 10 p.c. c-kit⁺ AGM or liver cells.
These data suggest that HSC activity peaks in the AGM region
at day 11 p.c. while this activity is increased enormously
in the liver at day 12 p.c.

c.   Comparative characterization of c-kit⁺ subsets in day 11 p.c. AGM and liver.

Previously, c-kit has been shown to be expressed not only
in HSCs but also in committed hematopoietic progenitors
cells and non-hematopoietic cells. Most notably, it has
been shown to be expressed on primordial germ cells (PGC)
located in the genital ridges of the AGM region. To
determine whether AGM and liver c-kit⁺ cells exhibited any
characteristics of these other populations, the expression
of lineage-specific surface antigens was performed by flow
cytometry. Expression analysis of surface antigens
associated with differentiated hematopoietic lineages showed
no significant levels of CD2, B220, CD8, CD4 and Gr-1 on the

surface of day 11 p.c. AGM and liver cells (data not shown).
However as shown in Figure 9A, Mac-1 was present in a
significant fraction of c-kit$^+$ cells in the AGM region (11%)
and liver (22%).   In both tissues, no significant co-
expression with c-kit was observed for the erythrocyte
associated antigen TER119 or Thy-1 (although Thy-1$^+$ cells
represent 14% of c-kit$^-$ AGM cells).   HSC associated antigens
Sca-1, AA4.1, CD34 and CD44 were also analysed.   Sca-1$^+$
cells represent less than 1% of the c-kit$^+$ population in the
AGM region and the liver. However, 20% of AGM c-kit$^+$ cells
and 94% liver c-kit$^+$ cells express high levels of CD44.   In
contrast with the liver where most c-kit$^-$ cells are CD44$^+$,
the c-kit$^-$ population of the AGM is CD44$^-$.   CD34, another HSC
associated antigen is expressed on 20% of c-kit$^+$ AGM cells
and on 37% of c-kit$^+$ liver cells.   Finally, AA4.1 which is
found on HSCs as well as pre-B cells is expressed on 18% of
c-kit$^+$ AGM region cells and 8% of c-kit$^+$ liver cells.

As c-kit is also expressed in primordial germ cells, the
c-kit$^+$ cells of the AGM region and liver were analysed for
expression of the enzyme alkaline phosphatase (AP).
Previously, AP has been used as a specific marker for
primordial germ cells.   As shown in Figure 9B, cytospin
preparations of AGM c-kit$^+$ sorted cells showed 15% AP$^+$ cells
(top panel) and 0% AP$^+$ cells in the liver c-kit$^+$ sorted cells
(bottom panel).   In the AGM c-kit^{lo} sorted population, 2-3
% AP$^+$ cells were detected (data not shown).

In general, the comparative phenotypic characterization of
c-kit$^+$ sorted cells from the AGM region and liver shows no
major differences between the hematopoietic antigens
expressed in these two anatomically distinct sites.
However, there are some differences in the percentage of
HSCs found within these sites which may be due to 1)
expansion of the c-kit committed progenitors within the
liver which decrease the overall percentage of HSCs and/or
2) the co-sorting of PGCs within the c-kit$^+$ AGM population.
Thus, the specific tissue localization of c-kit$^+$ cells
within the embryo appears to be a critical factor in the
assessment of HSC frequency and potential.

d. Expression of HSC cell markers in early- and mid-gestation embryos

CD34 has been previously used to enrich for HSCs in the adult mouse bone marrow. To determine whether CD34 expression may discriminate a c-kit$^+$ subpopulation with HSC activity in the day 11 p.c. AGM region and liver, transplantation studies were performed with c-kit and CD34 stained and sorted cells. As shown in Figure 3A, sorting gates were set for c-kit$^+$ CD34$^+$ (R1) and c-kit$^+$ CD34$^-$ (R2) subpopulations from the AGM and liver and varying numbers of cells injected into irradiated recipients. Peripheral blood DNA was analysed for the presence of the donor Lac-Z marker in these recipients at 6-10 months post-transplantation. The cumulative results of eight different experiments with 3-7 x 10^3 injected c-kit$^+$ CD34$^+$ AGM cells yielded 8 out of 26 (31%) recipient animals repopulated, whereas only 1 out 24 (4%) recipients were reconstituted with 3-10 x 10^3 injected c-kit$^+$ CD34$^+$ liver cells. If the number of c-kit$^+$CD34$^+$ liver cells are increased to 15-100 x 10^3, 5 out of 18 animals are reconstituted (28%). No HSC activity was found in 18 recipients transplanted with 3 - 10 x 10^3 c-kit$^+$ CD34$^-$ sorted AGM cells. However, 2 out of 9 and 1 out of 27 recipients injected with approximately 10 fold more c-kit$^+$ CD34$^-$ AGM or liver cells respectively were positive. It is uncertain whether this represents contamination by CD34$^+$ cells or true repopulating potential by CD34$^-$ cells. Cell lineage reconstitution experiments performed on 7 animals repopulated with AGM c-kit$^+$ CD34$^+$, 4 animals repopulated with liver c-kit$^+$ CD34$^+$ and one animal with liver c-kit$^+$ CD34$^-$ that were positive 6-8 months post-transplantation demonstrated that all hematopoietic tissues and lineages were donor cell derived. Levels of repopulation of splenic B cells, peritoneal macrophages, blood, thymus, spleen and lymph node were found to be 100% positive (data not shown) in 10 of the recipient animals analysed but two of them are 10% repopulated in macrophages and bone marrow. Thus, these studies demonstrate that HSC activity is found predominantly in the c-kit$^+$ CD34$^+$

subpopulation day 11 p.c. AGM and liver.

At day 12 p.c. the same phenotypic characteristics are observed (data not shown). CD34 is found on 60% of the c-kit$^+$ liver cells and the HSC activity resides completely in the c-kit$^+$ CD34$^+$ population. None of the 11 recipients transplanted with 10-30 x 10^3 c-kit$^+$ CD34$^-$ cells were reconstituted. Only 103 cells were required to yield 4 out of 8 recipients repopulated. Thus, HSCs are more enriched in day 12 p.c. c-kit$^+$ CD34$^+$ sorted liver cells than in th comparable day 11 sorted population. Thus, organism developmental time is a critical factor in the assessment of HSC potential within a phenotypically defined population.

e. Expression of Mac-1$^+$ in c-kit$^+$ CD34$^+$ AGM HSCs

To determine whether a distinctive embryonic marker could be found on AGM and early fetal liver HSCs, such cells were examined for the expression of Mac-1. Previously, it has been shown that in the day 13 p.c. liver, all HSCs are Mac-1$^+$. Thus, triple staining was performed with c-kit, CD34 and Mac-1 specific antibodies on day 11 p.c. AGM region and liver. Whereas most (81%) liver c-kit$^+$ CD34$^+$ cells are Mac-1$^+$, a smaller fraction (60%) of AGM region cells c-kit$^+$ CD34$^+$ are Mac-1$^+$. Transplantation experiments were performed with Mac-1$^+$ and Mac-1$^-$ sorting criteria (Figure 11). Both AGM subpopulations were found to contain HSCs in similar frequencies (Table 4). In contrast, most of the HSC activity in the day 11 p.c. liver is found within the c-kit$^+$ CD34$^+$Mac-1$^+$ population. This data is in accordance with the presence of approximately 2 times more c-kit$^+$ CD34$^+$ Mac-1$^-$ cells in the AGM than in the liver and is suggestive of Mac-1 as an ontogenic marker in the maturation pathway of HSCs and/or as an adhesion/homing molecule in determining the anatomical location of HSCs in development. Accordingly, using standard cell sorting techniques and one or more antibodies such as anti-c-kit, anti-CD 34 or anti-Mac-1, it is possible to obtain an enriched culture of HSCs.

EXAMPLE 3

Use of the *in vitro* culture of inducible and/or expandable HSCs in assays for identifying expansion and/or inducing

factors, and the cognate receptors.

a.   Expression cloning of novel hematopoietic stem cell
expansion and/or inducing factors   and cloning of the
cognate receptors.

In the hematopoietic system, growth factors and cytokines
factors signals hematopoietic cell proliferation and
differentiation through cell surface receptors. While many
hematopoietic factors and cytokines have been found to act
on hematopoietic progenitor cells of the adult bone marrow,
none have been found to specifically lead to the
proliferation of hematopoietic stem cells without
differentiation. However, HSC *in vitro* culture of the
present invention gives a unique opportunity to isolate
novel inducing and/or expansion factors for hematopoietic
stem ells. It has been found that during a small window of
developmental time (days 10 through 13 in mouse gestation)
hematopoietic stem cells increase in number in cultured AGM
region. Accordingly, it is possible to use the HSC culture
of the present invention to clone cell surface and secreted
molecules involved in hematopoietic stem cell induction and
expansion.

Previously, numerous groups have produced phage antibody
display libraries to isolate single-chain Fv antibodies
specific for cells of interest. Using the phage antibody
display system, it is possible to isolate antibodies having
affinity for HSCs and to use the antibodies to isolate
proteins produced by HSCs or receptors on the surface of
HSCs or which have an effect on HSC maturation or
proliferation. Such a library has been used for isolating
antibodies specific for human B cells, T cells and
eosinophils from peripheral blood (de Kruif et al., 1995a,
J. Mol. Biol., 248: 97-105; de Kruif et al., 1995b, Proc.
Natl. Acad. Sci. U.S.A., 92: 3938-3942), and may also be
used according to the invention to isolate antibodies
specific for AGM region, particularly the anterior portion
of the AGM which contains the pro/mesonephros and dorsal
aorta. In the previous experiments, it has been shown that
this portion is responsible for the generation of high level

hematopoietic stem cell activity. Furthermore in other experiments using transgenic mice expressing marker genes in hematopoietic stem cells, expression has been seen in thes sites (the pro/mesonephric tubules and cells surrounding the aorta (unpublished observations) of day 10-13 embryos). A phage antibody library is tested on frozen sections of anterior AGM tissue. To eliminate background, the phage antibody library is pre-absorbed on day of 9 AGM region which does not yet contain hematopoietic stem cells. After specific binding to day 10-13 AGM, individual phage are eluted and tested on day 11-13 liver. However, because hematopoietic stem cells from the AGM region appear to colonize the liver and continue to expand at day 11 and because the liver rudiment is derived from a different germ cell layer than the AGM region, only phage specific for the surface molecules of hematopoietic stem cells should bind. The specific phage is grown in large quantities. Phage antibody is then used in HSC culture experiments. If such antibodies are specific for hematopoietic stem cell surface receptors functioning in the induction and/or expansion of these cells, inhibition of stem cell production and no hematopoietic repopulation should be observed after transplantation of HSCs cells into irradiated recipient mice. The antibody is then used in Western blot analysis to isolate the specific cell surface molecules expressed on HSCs. The isolated proteins are sequenced and the deduced oligonucleotide sequences generated for PCR cloning of the genes from cDNA libraries. These libraries are generated from sorted day-11 AGM hematopoietic stem cells. Human homologues are subsequently cloned.

Simultaneously, the expansion and/or inducing factors to the cell surface receptors are cloned. An absorption of the phage antibody library on sorted AGM hematopoietic stem cells is first performed. The phage antibodies that remain unbound are incubated with sections of anterior AGM taken from culture. Selected phage antibodies are isolated and grown. Inhibition of hematopoietic stem cell production in HSC cultures by the individual phage antibodies is tested.

Thos that inhibit stem cell production are then used to isolate the specific inducing/expansion factors. Protein sequencing and gene cloning are perform d, and the genes are overexpressed in order to produce large quantities of these proteins for use in the culture experiments. The activity of the proteins in the induction of hematopoietic stem cells in day 9 and 10 AGM region is then tested. The activity on CD34[*] cells from human bone marrow and fetal liver is also tested.

b. RT-PCR or RDA Cloning of HSC expansion and/or inducing factors.

Another type of cloning can be performed using the cultured material. This is an RT-PCR differential display method. This method allows a relatively rapid detection of differentially expressed genes, including transcripts of low abundance (Liang et al., 1992, Science, 257: 967-971; Guimaraes et al., 1995a, Nucleic Acids Res., 23: 1832-1833; Guimaraes et al., 1995b, Development, 121: 3335-3346). A further enrichment for differentially-expressed transcripts of specific gene families may be obtained through the use of specific oligonucleotide primers for highly conserved domains such as the zinc fingers of transcription factors or tyrosine kinase domain of membrane receptors. Day 9 AGM region RNA are taken and compared to day 11 AGM RNA using the differential display method. The unique clones are then compared to day 11 liver RNA, which should contain some similar clones since hematopoietic stem cells are present and expanding in both tissue at this time. These clones are isolated and the full length cDNA cloned and sequenced. The function of the resulting molecules is tested in the culture as described above and on CD34+ cells from human bone marrow.

Another protocol which is of use in cloning genes that encode factors produced by HSCs is representational display analysis (RDA). This procedure, which entails a PCR-coupled subtractive cloning procedure, was developed for the comparison of complex genomes (Lisitsyn et al., 1993, Science, 259: 946-951), but has been adapted for

amplification of cDNA so that differentially-expressed genes may be xamined and cloned (Hubank and Schatz, 1994, _Nucleic Acids Res._, 22: 5640-5648). cDNA may be prepared by methods well known in the art from HSCs generated and/or expanded according to the methods of the invention.

Overall, the culture method facilitates both the cloning of novel genes as well as testing the activity of cloned genes and novel expansion/inducing factors.

EXAMPLE 4

Induction of hematopoietic stem cells in chimeric AGM cultures.

As shown above, E10 AGM explants autonomously and exclusively initiate hematopoietic stem cells and increase their number. To test whether such AGM explants could be used to induce/expand hematopoietic stem cells from exogenous sources, a chimeric culture system was developed. In principle, the recipient tissue is conditioned so as to eliminate developing cells of interest but not the cells of the microenvironment. Exogenous test cells (containing some distinguishing molecular marker) are then introduced into the (unmarked) ablated tissue. The chimeric tissue is cultured and subsequently tested qualitatively and quantitatively for the generation of marked cells of th desired cell type. The chimeric culture protocol for the generation of hematopoietic stem cells is as follows:

E11 AGM explants (which are highly active in the initiation of hematopoietic stem cells) were treated with doses of gamma irradiation ranging from 1000 to 0 Rads to eliminate endogenous hematopoietic stem cell activity but not the cells of the microenvironment. These treated (or "purged") E11 explants were injected with exogenous cells isolated from mouse embryonic day 9 (E9) yolk sac or AGM of transgenic (line 72; integration at β-globin locus) embryos. E9 tissues were used, since no hematopoietic stem cell activity can be detected either in E9 yolk sac or AGM cells explants directly transplanted into recipients or in untreated E9 yolk sac or AGM cultures. After two days of culture, the chimeric AGM tissues were harvested and

digest d with collagenase and single cell suspensions (.75 embryo equivalents) were injected into lethally-irradiated adult mouse recipients (CBAxB10 F1 mice; 900 Rads) to test for hematopoietic stem cell activity.

A Southern blot (Figure 12) of semi-quantitive PCR analysis performed on peripherial blood DNA obtained 1 month post-transplant from mouse recipients of chimeric AGM cultures showed that in six out of ten recipients injected with either E9 yolk sac (lanes 3, 4 and 7) or AGM cells (lanes 1, 2, 5, 6, 8, 9 and 10) cultured in E11 AGM explants, cells derived from the chimeric culture contributed to hematopoiesis in the adult recipients. Recipient AGM explants irradiated before injection with 1000 Rads (lanes 1 and 2), 750 Rads (lanes2 - 5), 500 Rads (lanes 7, 8 and 9) or 0 Rads are also shown.

The percentage repopulation by donor cells was determined by comparing the amount of β-globin signal in each sample with that of controls representing 100%, 10%, 1%, 0.1% and 0% β-globin DNA. Normalization of DNA was performed with myogenin. The Southern blot was hybridized with human β-globin and myogenin radiolabeled probes and quantiation was performed on a Phosphorimager. The levels of repopulation range from 5% to less than 0.1% at 1 month post-transplantation. Specifically, the percentage donor contriburion in lane 2 = 3-5%, lane 8 = .05% and lanes 6, 9, 4 and 7 less than 0.1%. At three months post-transplantation these recipients remain positive for the donor cell marker, indicating the engraftment is long-term. Thus, the chimeric cultures can induce and expand hematopoietic stem cell activity from exogenous sources thought to contain pre-hematopoietic stem cells.

While this level of population is low, other conditioning treatments for the recipient AGM explants aimed at optimizing repopulation may be used, including the use of AGM explants from mutant embryos known to be defective in hematopoietic stem cells. Additionally, the use of E10 AGM explants in the chimeric culture system, which may provide a more active microenvironment for the induction/expansion

of the hematopoietic stem cells, as well as enrichment of cells by flow cytometric procedures, since only a small volume can be inject d efficiently, may also be advantageous. Finally, it is contemplated that exogenously added growth factors and/or extension of the chimeric culture period will improve the efficiency of repopulation in recipients of the cultured cells (see below and Figure 13).

EXAMPLE 5

Transfection of Hematopoietic Stem Cells

HSC cultures obtained according to the invention are transfected with a gene of interest, and the transfected cells administered to a recipient mammal in need of the transfected gene of interest. Transfection of hematopoietic stem cells are described in Mannion-Henderson et al., 1995, Exp. Hematol., 23: 1628; Schiffmann et al., 1995, Blood, 86: 1218; Williams, 1990, Bone Marrow Transplant, 5: 141; Boggs, 1990, Int. J. Cell Cloning, 8: 80; Martensson et al., 1987, Eur. J. Immunol., 17: 1499; Okabe et al., 1992, Eur. J. Immunol., 22: 37-43; and Banerji et al., 1983, Cell, 33: 729. Administration of transfected cells is described below.

EXAMPLE 6

Generation of enriched populations of human HSCs according to the invention

In humans, there are many instances in which it would be advantageous to have a ready supply of HSCs for the purposes of therapeutic administration. These include both naturally-occurring blood cell deficiencies and those induced by radical treatment for other disorders, such as leukemia or other cancers. As previously stated, there is significant homology of the hematopoietic systems among mammals. Animal models, such as the murine culture system of the present invention, provide insight into human developmental hematopoiesis and therapeutic applications.

The expansion of a human myeloid leukemic cell in a mouse has been described previously (Lapidot et al., 1994, Nature, 367: 645-648). A colony of mice (scid/nod), which are

immunologically deficient, tolerate xenografted tissue
without subjecting it to immune rejection; therefore, AGM
tissue harvested from such mice may be co-cultured with
human HSCs that have been pre-enriched by flow-cytometric
sorting using antibodies directed at CD34 according to the
methods described above and shown in Figure 1. After such
cells are allowed to proliferate, quantitation of HSC
generation and/or expansion may be performed by
transplanting AGM cells or tissue into a recipient *scid/nod*
mouse and monitoring repopulation of that immune-deficient
recipient with human hematopoietic cells, as described in
the above Examples; the cells of the AGM to be used for
quantitation may be dissociated prior to transplantation,
if desired. For human transplantation, the *scid/nod* AGM
tissue is digested after culturing with collagenase and the
HCSs are again sorted out using an anti-CD34 antibody.
Unlike the example described above, in which mouse-AGM-
cultured HSCs are to be transplanted into a recipient mouse,
cell sorting is highly recommended prior to transplantation
into humans in order to avoid an immune response to the
mouse AGM cells. In a case in which the cells are to be
used to express a therapeutic transgene in a recipient
human, the procedures described in Example 5 may be followed
prior to transplantation.

The transplantation of hematopoietic cells, such as in a
bone marrow transplant, is commonly performed in the art by
procedures such as those described by Thomas et al. (1975,
New England J. Med., 292: 832-843) and modifications
thereof. Such a procedure is briefly summarized: In the
case of a syngeneic graft or of a patient suffering from an
immunological deficiency, no immunosuppressive pre-treatment
regiment is required; however, in cases in which a cells of
a non-self donor have been cultured according to the method
of the present invention for administration to a patient
with a responsive immune system, an immunosuppressive drug
must be administered, e.g. cyclophosphamide (50 mg/kg body
weight on each of four days, with the last does followed 36
hours later by the transplant). Leukemic patients routinely

receive a 1000-rad midline dose of total-body irradiation in order to ablate cancerous blood cells; this irradiation also has an immune suppressive effect. Following pre-treatment, HSCs generated in the *in vitro* culture system of the present invention are administered via injection, after which point they colonize the hematopoietic system of the recipient host. Success of the graft is measured by monitoring the re-appearance of the numerous adult blood cell types (see Summary, above) by the immunological and molecular methods described in the above Examples. While as few as 1-10 HSCs are able to colonize and repopulate a lethally-irradiated recipient mouse over time, it is advantageous to optimize the rate at which repopulation occurs in a human HSC transplant patient; therefore, 10 to 100, or even 100 to 1000 HCSs produced by the methods of the present invention should be administered in order to be therapeutically effective.

EXAMPLE 7

Long-term hempatopoietic stem cell growth in AGM explants.

Since the AGM region is a rapidly-growing embryonic area which changes in size, complexity and function from E10 to E13, it is of great importance to determine the viability and function of this microenvironment for the induction/growth/expansion of hematopoietic stem cells over long periods of time. Thus, we have cultured marked AGM explants for a period greater than 2-3 days and tested for the presence of hematopoietic stem cells by the long term adult repopulation assay. When E11 AGM explants marked with a transgene (β-globin locus, line 72) were cultured for 3 weeks, they were found to contain high levels of long-term-repopulating hematopoietic stem cells. Tissue harvesting, digestion and transplantation were performed as above; however, each recipient was injected with one embryo equivalent of AGM cells. A Southern blot of semi-quantitive PCR analysis performed on peripheral blood DNA obtained from mouse transplant recipients is shown in Figure 13, in which two out of four adult recipient mice at one month (lanes 1, 2, 3, and 4) and three months (lanes 5, 6, 7 and 8) post-

transplantation are shown to b long-term repopulated by donor cells derived from the AGM explants. The Southern blot was hybridized with human β-globin- and myogenin-radiolabeled probes and quantitation was performed on a Phosphorimager.

The percentage repopulation by donor cells was determined by comparing the amount of β-globin signal in each sample with that of controls representing 100%, 10%, 1%, 0.1% and 0% β-globin DNA. Normalization of DNA was performed using myogenin. The percentage donor contribution in lanes 1, 5 and 8 represent 100% donor contribution and lane 4 = 80% donor contribution. Limiting dilution experiments are currently in progress to determine the number of hematopoietic stem cells in the AGM explants after varying times in culture so as to determine the length of time during which the AGM explant remains active for induction/expansion of hematopoietic stem cells.

EXAMPLE 8

Preparation of AGM cells for the support of HCS culture

While intact AGM tissue, whether derived from the entire region or from the anterior or posterior regions, is of use according to the methods of the invention, it is also advantageous to dissociate the tissue into its component cells prior to culturing. In such experiments, reaggregation of dissociated cells, whether alone or mixed with exogenously-derived HSCs and/or pre-HSCs, into a solid or semi-solid mass has been observed to take place, and generation and/or expansion of HSCs has occurred in such cultures, as determined by the methods described above for the monitoring of HSC proliferation in intact- or anterior AGM cultures.

It is also advantageous to establish a culture system according to the invention using cells derived from AGM tissue. In order to do this, AGM tissue is digested with collagenase, as described above, and cells are seeded into tissue culture flasks or dishes in an appropriate medium, also as described above, and cultured. Foci are individually harvested and cloned by standard methods;

following establishment and expansion of a number of such cell lines, the candidate clones are mixed with HSCs and/or pre-HSCs, resulting chimerae ar cultured simlilar to intact AGM tissue, and the HSCs generated by the cultures quantitated either by immunological staining or by repopulation of lethally-irradiated mice, all as described above. Clones that support HSC generation and/or expansion are then retained for further use.

USE AND ADMINISTRATION

HSCs produced according to the invention are useful for therapy, such as bone marrow transplants, gene therapy and for the production of blood related proteins either in *in vivo* or *in vitro* production systems.

For therapies such as bone marrow transplants, a culture of HSCs or an enriched culture of HSCs is prepared according to the invention, for example, wherein the culture contains 0.1%-1.0% HSCs, is administered to a mammal in need thereof in a biocompatible manner (i.e., wherein the HSCs administered are able to repopulate the recipient blood system), and in an amount which suffices to treat the disorder in the mammal which has reduced or compromised the mammal's blood system, as described below.

For therapies such as gene therapy, a culture of HSCs or an enriched culture of HSCs is prepared as described above via transfection of the HSCs with a gene of interest. Stem cell transfection techniques are described above. A therapeutic gene is one which is expressible in a mammalian, preferably a human, cell and encodes RNA or a polypeptide that is of therapeutic benefit to a mammal, preferably a human. Examples of such genes are well known in the art and include but are not limited to the ß-glucocerebrosidase gene, the Bruton's thymidine kinase gene, genes encoding cytokines, such as TNF, interleukins 1-12, interferons (α, ß, γ), Fc receptor, and T-cell receptor. Other examples include genes encoding inhibitors of HIV, e.g., TAT or REV mutants that act as competitive inhibitors of the natural proteins. The gene of interest may be carried in a vector,

which vector may also includ marker genes, such as drug
resistance genes, th ß-galactosidase gene, the
dihydrofolate reductase gene, and the chloramphenicol acetyl
transferase gene.

Non-transfected and transfected HSCs are administered via
ex vivo gene therapy (see Anderson et al., U.S. Patent No.
5,399,346). The mode of administration is not critical to
the invention, and may include for example, administration
preferably in a biologically compatible solution or a
pharmaceutically acceptable delivery vehicle, by ingestion,
injection, inhalation or any number of other methods, i.e.,
via intravenous, intraperitoneal, and intradermal routes.

The dosages administered will vary from patient to patient;
a "therapeutically effective dose" will be determined by the
level of enhancement of function of the transferred genetic
material balanced against any risk or deleterious side
effects. Where the therapeutic gene encodes a product of
physiological importance, such as replacement of a defective
gene or an additional potentially beneficial gene function,
administration of transfected HSCs to a mammal in need
thereof is expected to confer long term genetic modification
of the cells and be effective in the treatment of disease.
The genetic modification and long term benefit may be
determined by detecting the gene product in the treated
mammal at various time points (i.e., several days or weeks
or months or years) after administration, or by detecting a
marker which indicates correction or amelioration of the
disease. Monitoring levels of gene introduction, gene
expression and/or the presence or levels of the encoded
product will assist in selecting and adjusting the dosages
administered.

Absolute numbers of HSCs used to repopulate an individual
substantially lacking hematopoietic cells may, in theory, be
as low as 1 to 10 in number; however, such a small number of
cells would require a lengthy period of proliferation and
differentiation before repopulation was effectively
complete. A thereapeutically useful number of HSCs should,
at a minimum, be 100 cells for a human recipient.

Generally, a composition according to the invention will be
administered in a single dose of preferably 100 to 1,000
cells, more preferably, 1,000 to 10,000 cells and most
preferably, up to 10,000 to 100,000 cells. If desired,
multiple doses may be administered, as needed.

Two representative genes of interest are described below
for treatment of human genetic diseases. HSCs provided
according to the invention may be used to treat X-linked
β-globulinemia. A selected vector containing the gene of
interest will contain the minimal sequences described
herein, i.e., an origin of replication for replication in a
bacterial or yeast host cell, an operator sequence, and a
site for insertion of a therapeutic gene. For example, the
pUC18tet plasmid may be used as a minimal plasmid,
preferably with the tet gene deleted. The therapeutic gene
may be the Bruton's kinase gene (Vetrie et al., 1993,
Nature, 361: 226-233), and is carried on the following DNA
fragments, which are cloned together using procedures
well-known in the art. The Bruton's Tyrosine Kinase human
gene is carried on a 2.1 kb fragment delineated by the PvuI
site at position (+33) and the HindIII site at position
(+2126). If desired, the vector also may include sequences
which confer position independent, tissue specific gene
expression, as taught in PCT/GB88/00655. The therapeutic
gene may also encode a splice site and poly-A tail, which
may include portions of the human β globin locus splice and
poly A signals; i.e., a BamHI - XbaI 2.8 kb 3' splice/poly-
A flanking sequence containing exon 2 - IVSII - exon 3 -
polyA sequences.

Vector DNA may be prepared as described herein and used to
treat X-linked β-globulinemia by introducing vector-
transfected HCSs for ex vivo therapy, and administering the
transfected cells into a patient afflicted with X-linked
β-globulinemia.

HSCs prepared according to the invention also may be used
for treatment of Gaucher's disease. Gaucher's disease stems
from one of two different genetic mutations. Gaucher's type
1 is a CGG --> CAG mutation, which results in an Arg --> Gln

substitution at position 119 of the ß-glucocerebrosidase polypeptide (Graves, 1988, DNA, 7: 521). Gaucher's type 2 is a CTG - -> CCG mutation, which results in a L u --> Pro substitution at position 444 of the ß-glucoc rebrosidase polypeptide (Tsuji, 1987, New England J. Med., 316: 570). The presence of a ß-glucocerebrosidase gene encoding a wild type polypeptide is believed to substantially correct Gaucher's disease. Therefore, another vector useful according to the invention for transfecting HSCs is one containing the minimal elements described herein (i.e., an origin of replication, an operator sequence, and a cloning site) and the lysozyme gene promoter and the ß-glucocerebrosidase transgene, as described in Horowitz et al., 1989, Genomics, 4: 87-96. This plasmid is constructed as follows.

The human ß-glucocerebrosidase gene is carried (as disclosed in Horowitz et al., 1989, supra) on a 9722 base pair fragment extending from a BamHI site in exon 1 to an EcoRV site 3' to polyadenylation site. This fragment contains 11 exons and all intervening sequences, with translational start in exon 2. Sequences conferring position-independent and tissue-specific gene expression may be included in the construct and are carried on an 11.8 kb XhoI - SacI fragment from pIII.lyx construct as described in Bonifer et al., 1990, EMBO J., 9: 2843.

DNA is prepared as described herein and is then used to treat Gaucher's disease by introducing the DNA into HSCs as described above and then administering the transfected HSCs into a patient afflicted with Gaucher's disease. Expression of the wild type transgene in a patient afflicted with Gaucher's disease should result in correction of the diseased state.

For the production of blood related proteins either in *in vivo* or *in vitro* production systems, HSCs are prepared according to the invention, whether untransfected or transfected with a gene of interest. *In vivo* protein production systems, for example, animals which carry a gene of interest, are well-known in the art. Thus, HSCs are

administered to an animal such as a mouse, pig, goat, or cow, in the same cell numerical range describ d above for diseas treatment, and the protein of interest (i.e., produced by the HSCs) is obtained from the animal by removing blood from the animal and isolating the protein of interest using techniques known in the art for isolation of that protein of interest. For *in vitro* protein production, a culture of HSCs is provided as described herein, and the protein is isolated according to standard techniques.

Table 1

Dev lopment f CFU-S_11 in organ cultur of day 10 p.c. tissues

Somite pairs	Tissue	CFU-S/ tissue	CFU-S/ 10^6 cells
32-33	Yolk sac	0.5	3.6
	AGM	4.8	86.4
	Head/heart	0	0
	Liver	0	0
	Body Remnants	0	0
34-35	Yolk sac	1	12.5
	AGM	9.3	53.8
	Head/heart	0	0
	Liver	0	0
	Body Remnants	0.3	13.0

Embryonic tissues were cultured as described in Figure 1 and tested for CFU-S_11 generation. The number of CFU-S per tissue and CFU-S per 10^6 cells are shown.   In two representative experiments, the total numbers of 32-33 and 34-35 somite pair embryos used for organ cultures were 4 and 3 respectively. Temporal dynamics (at day 10 and 11 p.c.) of the in vitro CFU-S_11 progenitor production is shown in Figure 3 for cultured YS, AGM region and liver.   In recipient mice transplanted with cultured body remnants we found some CFU-S production which likely represents contamination with the AGM region (as the AGM region is juxtaposed the somites and lateral walls of the embryo).

Table 2

LTR-HSC activity in organ cultur s of day 10 p.c. tissues

Tissue	Positive/Total at 2 months*	Positive/Total at 8 months
AGM	34/36	24/27
Yolk sac	0/16	0/16
Liver	0/10	0/10
Body remnants	0/7	0/4

The number of reconstituted, donor positive recipients and the total number of recipients transplanted with cultured embryonic tissues is shown. Tissues from day 10 p.c. embryos (35-38 somite pairs) were explanted to organ cultures as described in Figure 1. Only male tissues were transplanted into lethally irradiated female recipients. The number of reconstituted donor positive recipients was determined by TMT2/B specific PCR analysis of peripheral blood DNA at the post-transplantation times shown as described in the Materials and Methods. Semi-quantitative PCR showed the above number of recipients were reconstituted with greater than 10% donor cells. We also observed some reconstituted animals with about 1% donor contribution as follows: 3/27 with cultured AGM region, 1/16 with cultured YS and 1/4 with cultured body remnants. A small number of recipients were reconstituted with about 0.1% donor cells. The number of embryo tissue equivalents transplanted per recipient was 1-2.5 in 12 out of 13 experiments. In one experiment 3.7 embryo tissue equivalents was transplanted. *Not all transplant recipient animals were analysed at 2 months.

Table 3 R constitution with c-kit+ cells fr m 10, 11 and 12
dpc AGM and liver.

Embryo	tissue	cells per (x10³) §	embryo equivalents per recipient	reconstituted recipients/ total recipients
day 10	*AGM	5.7(0.5-14)	2.8(1.2-6)	0/18
	**Liver	7.4(7-12)	1.5(1.2-1.5)	0/15
*** day 11	AGM	8.4(1.7-20)	3(0.4-5)	18/32
	Liver	60.5(10-100)	3.3(0.4-5)	13/27
**** day 12	AGM	11.5 (5-28)	1.7(0.7-4)	8/20
	Liver	6(5-7)	0.1 (0.1-0.2)	13/14

Reconstitution data obtained after one month post transfer.
Positive recipients were identified by PCR amplification of
the donor Lac-Z signal from genomic DNA obtained from blood.
Level of reconstitution was determined as indicated in Table
2.   Only recipients with signal >10% are considered
positive.

§   The mean number of injected cells per recipient is
shown and the range is indicated in parentheses.
*   mean of four experiments. c-kit+ included c-kit+ and
        c-kit^{lo}.
**   mean of three experiments.
***   mean of 9 experiments.
****   mean of 3 experiments.

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Table 4 Charact rization of c-kit+ cells with long t rm
reconstitution activity obtained from 11 dpc embryonic AGM
region and liver.

tissue	cell type	number of experiments	donor cells per recipient (x 1000) *	reconstituted recipients/ total recipients **
AGM	c-kit+ CD34+	8	4.5 (3-7)	8/26
	c-kit+	8	6.4 (3-10)	0/27
	CD34-	3	46 (42-50)	2/9
Liver	c-kit+	8	7.1 (3-10)	1/24
	CD34+	8	50 (15-100)	5/18
	c-kit+	8	6.6 (3-10)	0/18
	CD34-	8	94 (25-300)	1/27
AGM	c-kit+ Mac-1+	5	4.1 (3.5)	3/17
	c-kit+	4	4.5 (4-5)	2/13
	Mac-1-	3	28 (18-33)	4/10
Liver	c-kit+	4	5 (4-7.5)	1/14
	Mac-1+	3	21 (15-30)	4/8
	c-kit+	4	5 (4-5)	0/10
	Mac-1-	7	80 (33-150)	1/19

Cells were stained with FITC-conjugated anti-c-kit and PE-
conjugated anti-CD34 mAbs or with FITC-conjugated anti-c-
kit and PE-conjugated anti-Mac-1 mAbs and sorted according
to the gates indicated in fig 3. Each sorted subset was
i.v. injected into irradiated recipients mice.

* The mean number of rejected cells per recipient is shown
and the range is indicated in parentheses.

** analysis after 6-12 months

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OTHER EMBODIMENTS

Other embodiments of the present invention will be evident to thos of skill in the art. It should be understood that the above exampl s are provided for exemplification only and the scope of the present invention is not limited to the examples above but is encompassed by the following claims.

CLAIMS

What is claimed is:

1.   An *in vitro* culture system comprising cells of the AGM of an embryonic mammal, and nutrients sufficient to support the expansion of mammalian HSCs and/or their generation from pre-HSCs, and containment therefor.

2.   The culture system of claim 1, wherein said cells comprise tissue.

3.   The culture system of claim 1 wherein said cells reside on a solid support.

4.   The culture system of claim 1, wherein said culture comprises colony-forming-unit spleen (CFU-S) cells.

5.   The culture system of claim 1, wherein said AGM is from a first mammal and said HSCs and/or pre-HSCs are from a second mammal.

6.   The culture system of claim 5, wherein said first and second mammals are a single individual of a single species.

7.   The culture system of claim 5, wherein said first and second mammals are different individuals of a single species and the AGM of said first mammal substantially lacks endogenous HSCs and pre-HSCs prior to the introduction of said HSCs and/or pre-HSCs from said second mammal in culture.

8.   The culture system of claim 5, where said first and second mammals are different individuals of two different species and the AGM of said first mammal substantially lacks endogenous HSCs and pre-HSCs prior to the introduction of said HSCs and/or pre-HSCs from said second mammal in culture.

9.   The culture system of claim 5, wherein said first mammal is a non-human mammal, said second mammal is a human, and the AGM of said first mammal substantially lacks endogenous HSCs and pre-HSCs prior to the introduction of said HSCs and/or pre-HSCs from said human in culture.

10.   The culture system of claim 9, wherein said first mammal is immune-deficient.

11.   The culture system of claim 5, wherein said HSCs and or pre-HSCs of said second mammal are transfected with a gene of interest.

12.   An *in vitro* culture method, comprising culturing cells from the AGM of a mammalian embryo *in vitro* under conditions which permit generation and/or expansion of HSCs.

13.   The method of claim 12, wherein said cells are cultured on a solid support.

14.   The method of claim 13, further comprising before said culturing step, the step of substantially purging said cells of said AGM of endogenous HSCs and pre-HSCs, contacting said purged cells with exogenous HSCs and/or pre-HSCs so as to form a mixture of cells, and culturing said mixture of cells.

15.   The method of claim 14, wherein said culturing step permits aggregation of said cells to form tissue.

16.   The method of claim 13 wherein said cells comprise tissue.

17.   The method of claim 15 or 16, further comprising the step after culturing of dissociating the cells of said tissue.

18.   The method of claim 17 wherein after the dissociating

step, the method further comprises the step of sorting HSCs
fr m said cells.

19.   The method according to claim 13, wherein said tissue
is cultured at the air/medium interface.

20.   The method according to claim 13, wherein said tissue
is cultured at 37°C, under 5% CO_2, in myeloid long-term
culture medium supplemented with hydrocortisone succinate to
a concentration of approximately 10^{-5}M to 10^{-6}M.

21.   The method according to claim 13, wherein said cells
of said AGM is taken from a mammal during the stage at which
hematopoietic stem cells are maximally proliferative in that
tissue.

22.   The method according to claim 1 or claim 13, wherein
said AGM comprises the anterior section of the AGM.

23.   A method of treating a mammal having a deficiency of
HSCs, comprising the steps of
   a) culturing cells of the AGM of an embryonic mammal *in
vitro* under conditions which permit generation and/or
expansion of HSCs to produce HSCs, and
   b) administering said HSCs of step a) to a mammal in need
thereof.

24.   The method of claim 23 wherein in said step a), said
cells comprise tissue.

25.   A method of identifying receptors on the surface of
HSCs, comprising generating and/or expanding HSCs using the
culture system of claim 1 or the method of claim 13, and
making a comparison of the receptors thereon with those on
cells of the hematopoietic system other than HSCs.

26.   The method according to claim 25, wherein said
comparison is made using the phage antibody display assay.

27. A method for identifying factors produced by HSCs, comprising expanding HSCs using the culture syst m of claim 1 or the method of claim 13, and making a comparison of the factors produced thereby with those produced by cells of the hematopoietic system other than HSCs.

28. The method of claim 27 wherein said comparison is performed using RT-PCR differential display assay.

29. The method of claim 27 where said comparison is performed using representational display analysis (RDA).

9 - 12 DAY EMBRYO

FIG. 1

YOLK SAC
AGM
LIVER
BODY REMNANTS

2 - 3 DAYS

ORGAN CULTURE

CELL SUSPENSION

LTR - HSC
ACTIVITY

CFUs
ACTIVITY
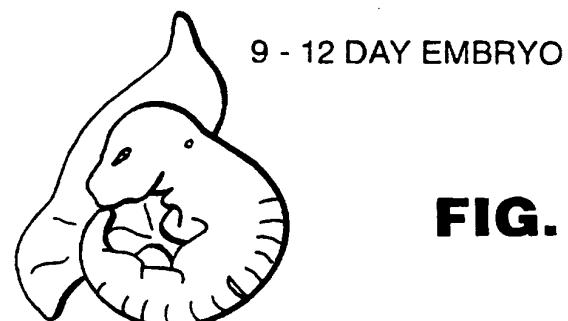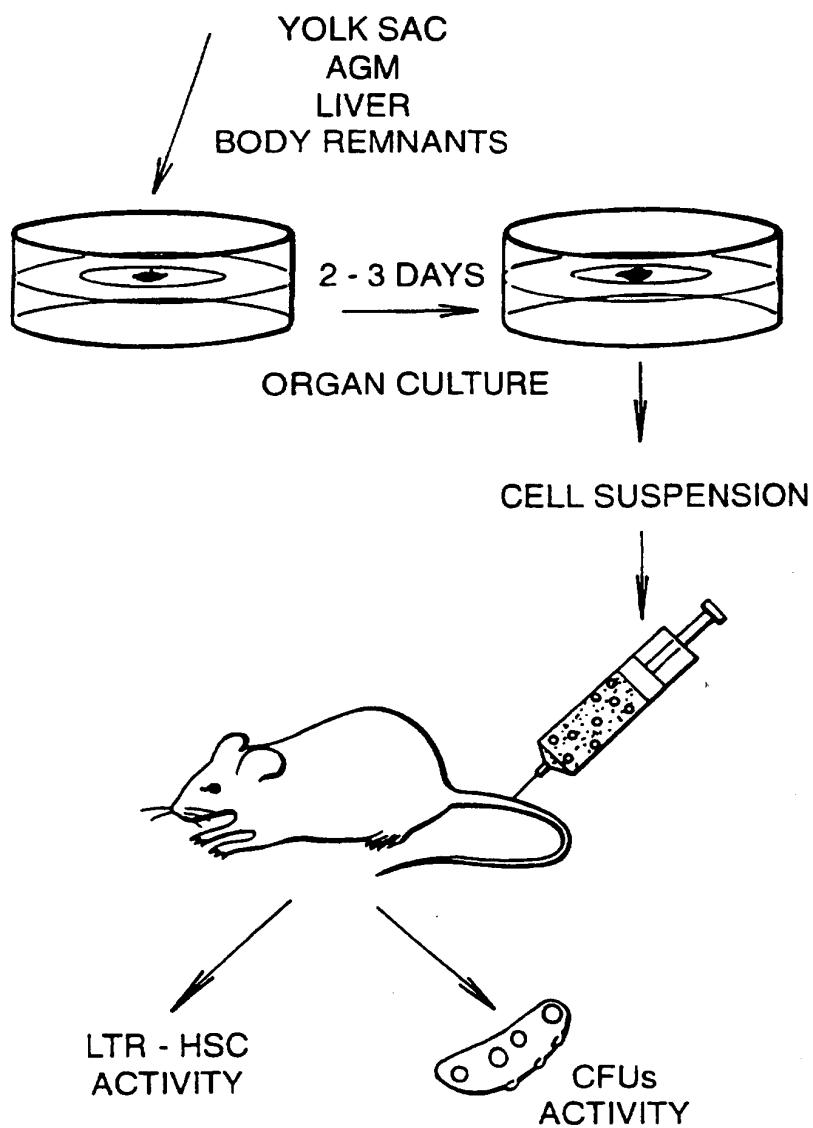
FIG. 2

Yolk Sac
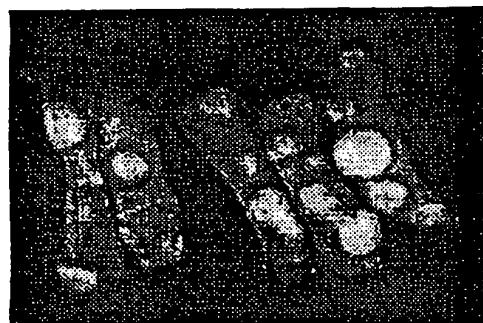
9.2±3.1 colonies/tissue
38.6±7.8 mg

AGM

31.4±5.7 colonies/tissue
94.4±12.2 mg

Liver

1.8±1.9 colonies/tissue
21.4±2.5 mg

Control
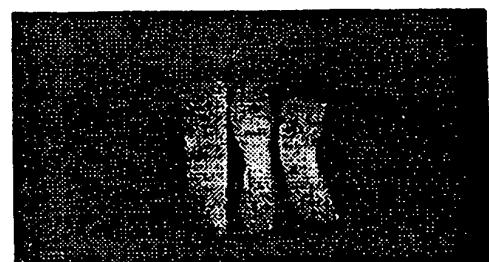
0 colonies/tissue
19.3±1.1 mg
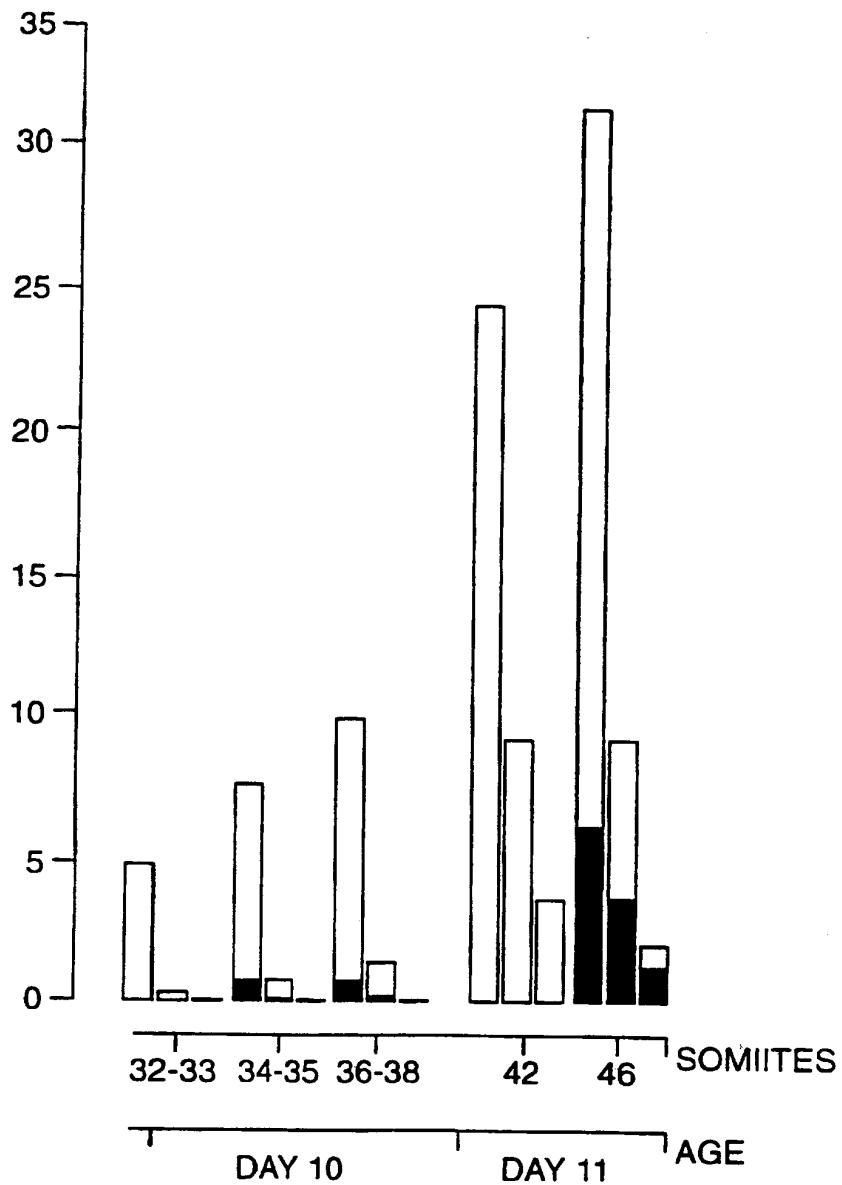
FIG. 3

UNCULTURED   CULTURED

■ AGM ▢

■ YS ▢

■ LIVER ▢

FIG. 4A

FIG. 4B

FIG. 5

FIG. 6A

Dorsal aorta
Pronephros
Mesonephros
Gonad

Anterior AGM / Posterior AGM

FIG. 6B

FIG. 7A

FIG. 7B

FIG. 7C

FIG. 7D

FIG. 8

FIG.9A

FIG.9A (CONTD.)

AGM
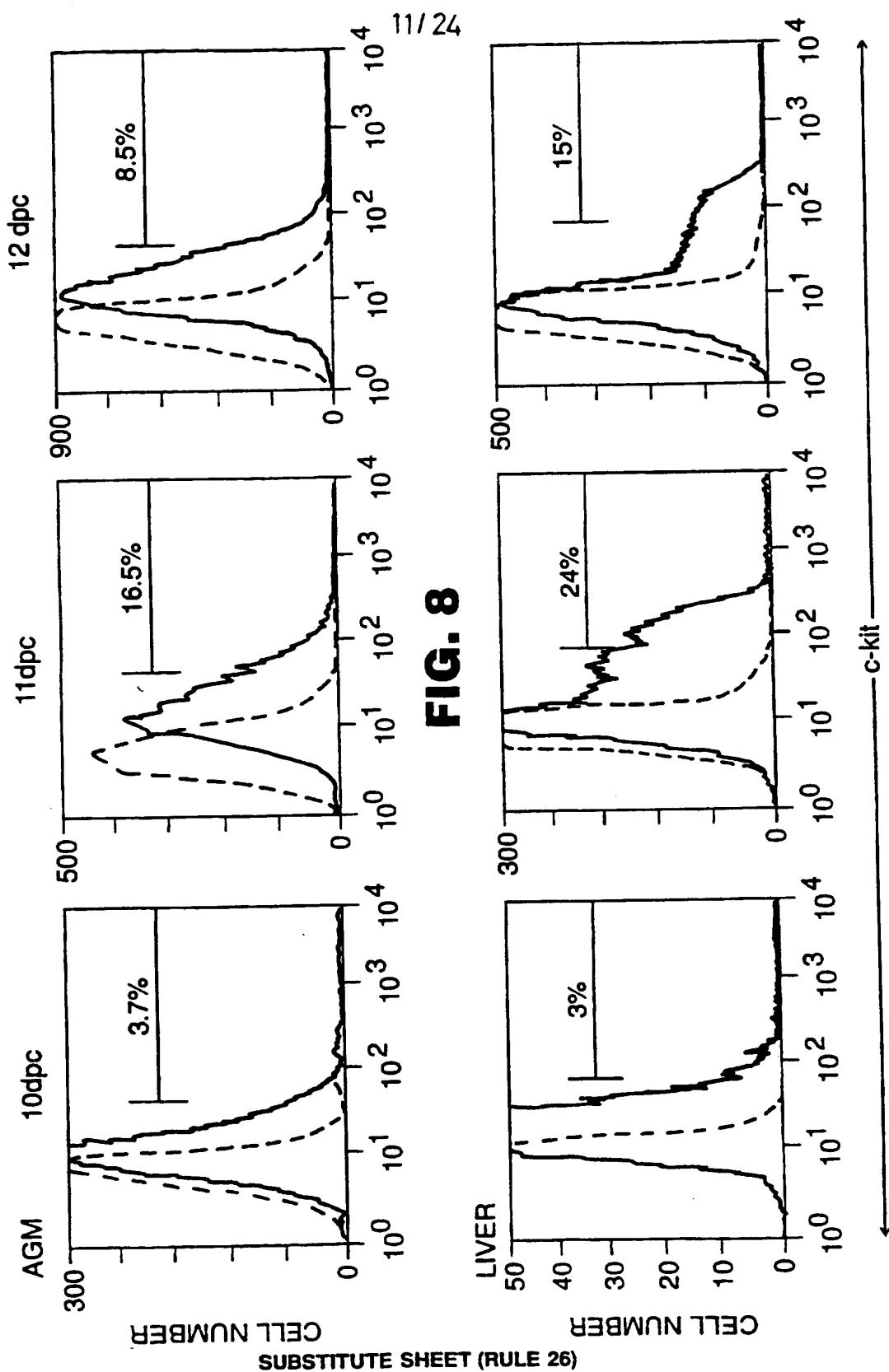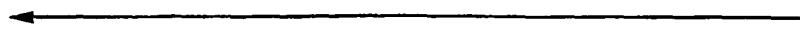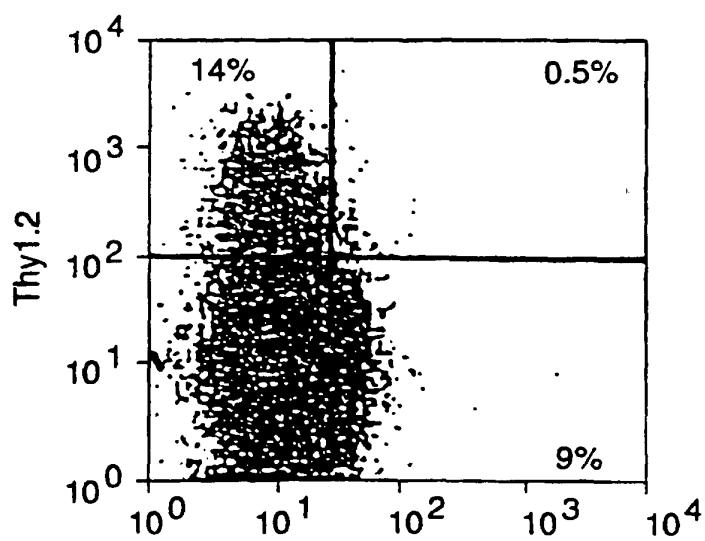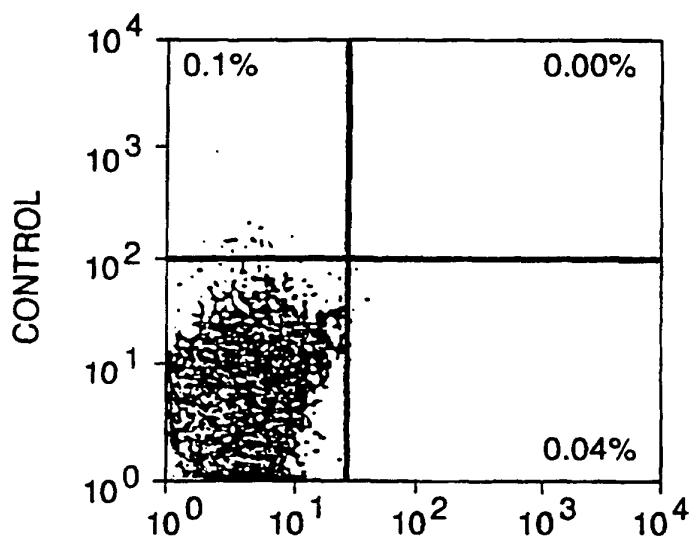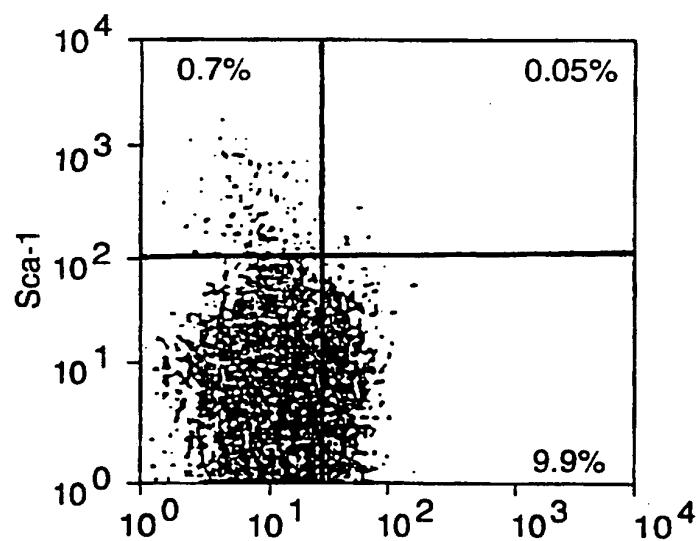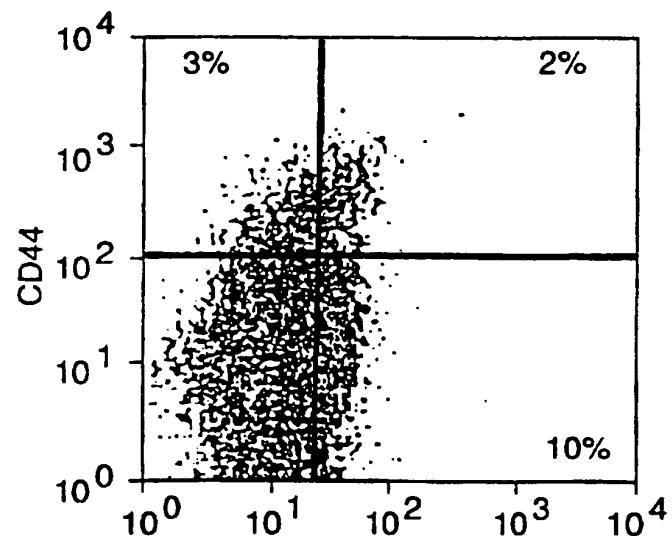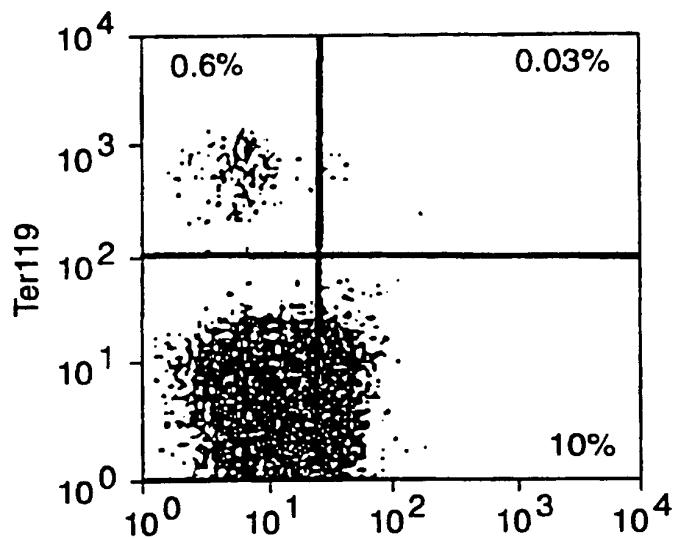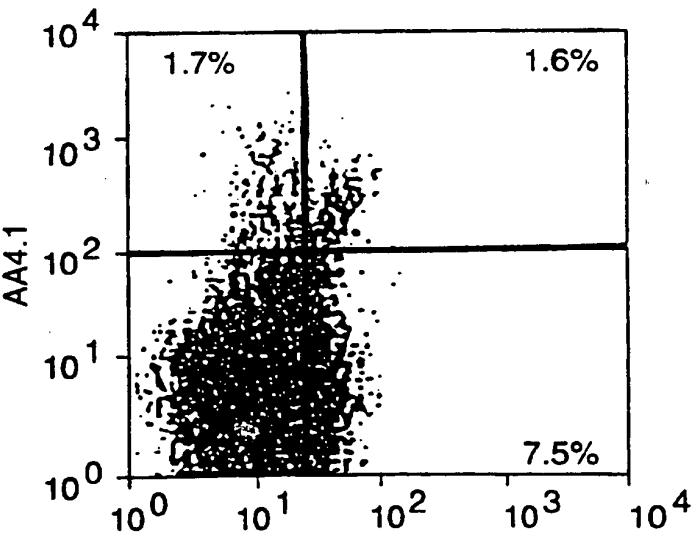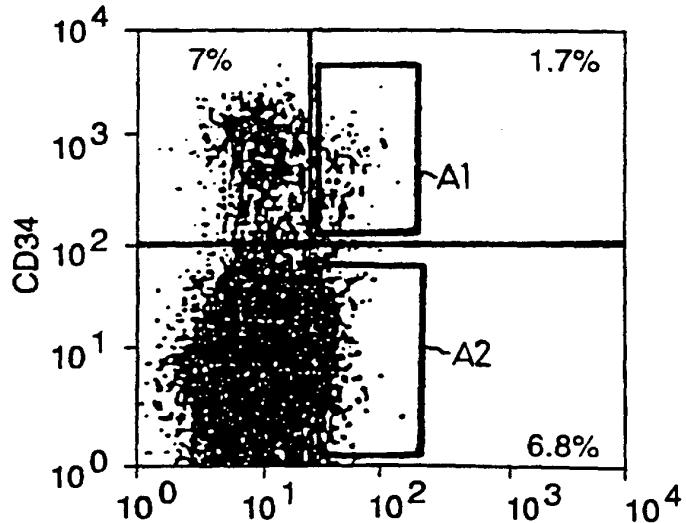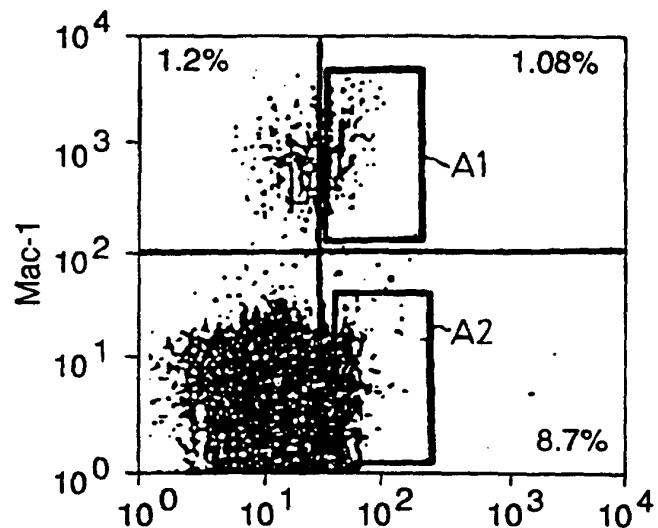
FIG.9A (CONTD.)

FIG.9A (CONTD.)

FIG.9A (CONTD.)

FIG.9A (CONTD.)

LIVER

FIG.9A (CONTD.)

LIVER

c-kit

FIG.9A (CONTD.)

LIVER

FIG. 9B

FIG.10

FIG.10 (CONTD.)

FIG. 11
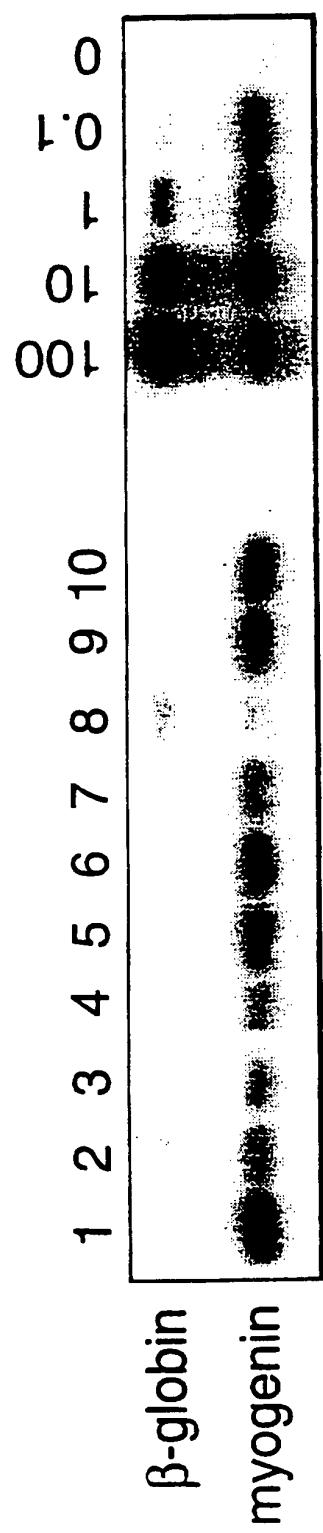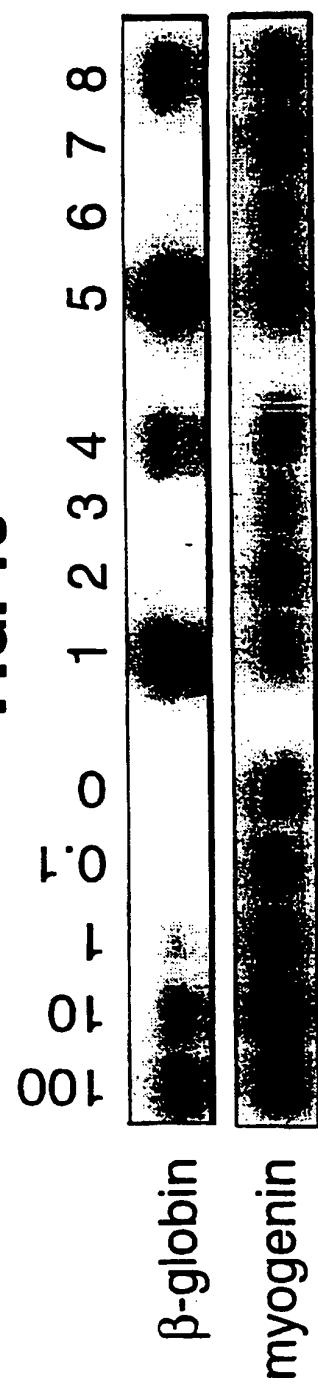
FIG. 12

FIG. 13

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N5/06   A61K35/28   C12Q1/68   G01N33/577   //A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12N   A61K   C12Q   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MEDVINSKY A L ET AL: "Development of day-8 colony-forming unit-spleen hematopoietic progenitors during early murine embryogenesis: Spatial and temporal mapping." BLOOD 87 (2). 1996. 557-566, XP002052535 cited in the application see the whole document ---	1-29
A	DZIERZAK E. ET AL.: "Mouse embryonic hematopoiesis" TRENDS IN GENETICS, vol. 11, no. 9, September 1995, pages 359-366, XP002052536 cited in the application see the whole document ---	1-29

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☒ Further documents are listed in the continuation of box C.   ☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 January 1998	1 7. 02. 98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

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INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED T BE RELEVANT		
Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	MUELLER, ALBRECHT M. ET AL: "Development of hematopoietic stem cell activity in the mouse embryo" IMMUNITY (1994), 1(4), 291-301, XP002052537 cited in the application see the whole document ---	1-29
P,X	DZIERZAK E ET AL: "Induction and expansion of hematopoietic stem cells in the AGM region of the developing mouse embryo." THIRTY-EIGHTH ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY, ORLANDO, FLORIDA, USA, DECEMBER 6-10, 1996. BLOOD 88 (10 SUPPL. 1 PART 1-2). 1996. 444A, XP002052538 see the whole document ---	1-29
P,X	MEDVINSKY A ET AL: "Definitive hematopoiesis is autonomously initiated by the AGM region." CELL 86 (6). 1996. 897-906, XP002052539 see the whole document ---	1-29
P,X	DZIERZAK E ET AL: "The AGM region: Initiation and characterization of the first definitive hematopoietic stem cells in the mouse embryo." 26TH ANNUAL MEETING OF THE INTERNATIONAL SOCIETY FOR EXPERIMENTAL HEMATOLOGY, CANNES, FRANCE, AUGUST 24-28, 1997. EXPERIMENTAL HEMATOLOGY (CHARLOTTESVILLE) 25 (8). 1997. 876, XP002052540 see the whole document ---	1-29
P,X	SANCHEZ, MARIA-JOSE ET AL: "Characterization of the first definitive hematopoietic stem cells in the AGM and liver of the mouse embryo" IMMUNITY (1996), 5(6), 513-525, XP002052541 see the whole document -----	1-29

INTERNATIONAL SEARCH REPORT

Box I   Obs rvations where certain claims were f und unsearchable (Continuation of Item 1   f first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

    see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
*because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:*

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

INTERNATIONAL SEARCH REPORT

FURTHER INF RMATION C NTINUED FROM . PCT/ISA/ 210

Remark : Although claims 23-24 are directed to a method of treatment of
the human/animal body , the search has been carried out and based on the
alleged effects of the compound/composition.